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<p>(54) Title: <b>CHIMERIC IMMUNOGENS</b></p> <p>(57) Abstract</p> <p>Multimeric hybrid genes encoding the corresponding chimeric protein comprise a gene sequence coding for an antigenic region of a protein from a first pathogen linked to a gene sequence coding for an antigenic region of a protein from a second pathogen. The pathogens particularly are parainfluenza virus (PIV) and respiratory syncytial virus (RSV). A single recombinant immunogen is capable of protecting infants and similar susceptible individuals against diseases caused by both PIV and RSV.</p>		

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CHIMERIC IMMUNOGENSFIELD OF INVENTION

5       The present invention relates to the engineering and expression of multimeric hybrid genes containing sequences from the gene coding for immunogenic proteins or protein fragments of numerous pathogens.

BACKGROUND TO THE INVENTION

10      The advantage of the approach taken by the present invention is to produce single immunogens containing protective antigens from a range of pathogens. Such chimeras greatly simplify the development of combination vaccines, in particular, with the view ultimately to 15 produce single dose multivalent vaccines. Multivalent vaccines are currently made by separately producing pathogens and/or their pertinent antigens and combining them in various formulations. This is a labour intensive, costly and complex manufacturing procedure.

20      In contrast, the availability of a single immunogen capable of protecting against a range of diseases would solve many of the problems of multivalent vaccine production. Several chimeric immunogens of the type provided herein may be combined to decrease the number of 25 individual antigens required in a multivalent vaccine.

Human Parainfluenza virus types 1,2,3 and Respiratory syncytial virus types A and B are the major viral pathogens responsible for causing severe respiratory tract infections in infants and young 30 children. It is estimated that, in the United States alone, approximately 1.6 million infants under one year of age will have a clinically significant RSV infection each year and an additional 1.4 million infants will be infected with PIV-3. Approximately 4000 infants less 35 than one year of age in the United States die each year from complications arising from severe respiratory tract disease caused by infection with RSV and PIV-3. The WHO

and NIAID vaccine advisory committees ranked RSV number two behind HIV for vaccine development while the preparation of an efficacious PIV-3 vaccine is ranked in the top ten vaccines considered a priority for vaccine 5 development.

Safe and effective vaccines for protecting infants against these viral infections are not available and are urgently required. Clinical trials have shown that formaldehyde-inactivated and live-attenuated viral 10 vaccines failed to adequately protect vaccinees against these infections. In fact, infants who received the formalin-inactivated RSV vaccine developed more serious lower respiratory tract disease during subsequent natural RSV infection than did the control group. [Am. J. 15 Epidemiology 89, 1969, p.405-421; J. Inf. Dis. 145, 1982, p.311-319]. Furthermore, RSV glycoproteins purified by immunoaffinity chromatography using elution at acid pH induced immunopotentiation in cotton rats. [Vaccine, 10(7), 1992, p.475-484]. The development of efficacious 20 PIV-3 and RSV vaccines which do not cause exacerbated pulmonary disease in vaccinees following injection with wild-type virus would have significant therapeutic implications. It is anticipated that the development of a single recombinant immunogen capable of simultaneously 25 protecting infants against diseases caused by infection with both Parainfluenza and Respiratory syncytial viruses could significantly reduce the morbidity and mortality caused by these viral infections.

It has been reported that a protective response 30 against PIV-3 and RSV is contingent on the induction of neutralizing antibodies against the major viral surface glycoproteins. For PIV, these protective immunogens are the HN protein which has a molecular weight of 72 kDa and possesses both hemagglutination and neuraminidase 35 activities and the fusion (F) protein, which has a molecular weight of 65 kDa and which is responsible for

both fusion of the virus to the host cell membrane and cell-to-cell spread of the virus. For RSV, the two major immunogenic proteins are the 80 to 90 kDa G glycoprotein and the 70 kDa fusion (F) protein. The G and F proteins  
5 are thought to be functionally analogous to the PIV HN and F proteins, respectively. The PIV and RSV F glycoproteins are synthesized as inactive precursors (FO) which are proteolytically cleaved into N-terminal F2 and C-terminal F1 fragments which remain linked by disulphide  
10 bonds.

Recombinant surface glycoproteins from PIV and RSV have been individually expressed in insect cells using the baculovirus system [Ray et al., (1989), Virus Research, 12: 169-180; Coelingh et al., (1987), Virology, 160: 465-472; Wathen et al., (1989), J. of Inf. Dis. 159: 253-263] as well as in mammalian cells infected with recombinant poxviruses [Spriggs, et al., (1987), J. Virol. 61: 3416-3423; Stott et al., (1987), J. Virol. 61: 3855-3861]. Recombinant antigens produced in these  
15 systems were found to protect immunized cotton rats against live virus challenge. More recently, hybrid RSV F-G [Wathan et al., (1989), J. Gen Virol. 70: 2625-2635; Wathen, published International Patent application WO 89/05823] and PIV-3 F-HN [Wathen, published International Patent Application WO 89/10405], recombinant antigens  
20 have been engineered and produced in mammalian and insect cells. The RSV F-G hybrid antigen was shown to be protective in cotton rats [Wathan et al., (1989), J. Gen. Virol. 70: 2637-2644] although it elicited a poor anti-G antibody response [Connors et al., (1992), Vaccine 10: 475-484]. The protective ability of the PIV-3 F-HN protein was not reported in the published patent application. These antigens were engineered with the aim  
25 to protect against only the homologous virus, that is either RSV or PIV-3. However, it would be advantageous and economical to engineer and produce a single  
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recombinant immunogen containing at least one protective antigen from each virus in order simultaneously to protect infants and young children against both PIV and RSV infections. The chimeric proteins provided herein for such purpose also may be administered to pregnant women or women of child bearing age to stimulate maternal antibodies to both PIV and RSV. In addition, the vaccine also may be administered to other susceptible individuals, such as the elderly.

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SUMMARY OF INVENTION

hybrid gene, which may be a viral vector or a bacterial vector, and a physiologically-acceptable carrier therefor. Such live vector may form the active component of a vaccine against diseases caused by multiple pathogenic infections. Such vaccine may be formulated to be administered in an injectable form, intranasally or orally.

In an additional aspect of the present invention, there is provided a process for the preparation of a chimeric protein, which comprises isolating a gene sequence coding for an antigenic region of a protein from a first pathogen; isolating a gene sequence coding for an antigenic region of a protein from a second pathogen; linking the gene sequences to form a multimeric hybrid gene; and expressing the multimeric hybrid gene in a cellular expression system. Such cellular expression system may be provided by bacterial cells, mammalian cells, insect cells, yeast cells or fungal cells. The chimeric protein product of gene expression may be separated from a culture of the cellular expression system and purified.

The present invention further includes a vaccine against diseases caused by multiple pathogen infections, comprising the chimeric protein encoded by the multimeric hybrid gene and a physiologically-acceptable carrier therefor. Such vaccine may be formulated to be administered in an injectable form, intranasally or orally.

The vaccines provided herein may be used to immunize a host against disease caused by multiple pathogenic infections, particularly those caused by a parainfluenza virus and respiratory syncytial virus, by administering an effective amount of the vaccine to the host. As noted above, for human PIV and RSV, the host may be infants and young children, pregnant women as well as those of a

child-bearing age, and other susceptible persons, such as the elderly.

The chimeric protein provided herein also may be used as a diagnostic reagent for detecting infection by 5 a plurality of different pathogens in a host, using a suitable assaying procedure.

It will be appreciated that, while the description of the present invention which follows focuses mainly on a chimeric molecule which is effective for immunization 10 against diseases caused by infection by PIV and RSV, nevertheless the invention provided herein broadly extends to any chimeric protein which is effected for immunization against diseases caused by a plurality of pathogens, comprising an antigen from each of the 15 pathogens linked in a single molecule, as well as to genes coding for such chimeric molecules.

In this application, by the term "multimeric hybrid genes" we mean genes encoding antigenic regions of proteins from different pathogens and by the term 20 "chimeric proteins" we mean immunogens containing antigenic regions from proteins from different pathogens.

#### BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows the nucleotide (SEQ ID No: 1) and amino acid (SEQ ID No: 2) sequence of a PCR-amplified 25 PIV-3 F gene and F protein, respectively;

Figure 2 shows the restriction map of the PIV-3 F gene;

Figure 3 shows the nucleotide (SEQ ID No: 3) and amino acid (SEQ ID No: 4) sequences of the PIV-3 HN gene 30 and HN protein, respectively;

Figure 4 shows the restriction map of the PIV-3 HN gene;

Figure 5 shows the nucleotide (SEQ ID No: 5) and amino acid (SEQ ID No: 6) sequences of the RSV F gene and 35 RSV F protein, respectively;

Figure 6 shows the restriction map of the RSV F gene;

Figure 7 shows the nucleotide (SEQ ID No: 7) and amino acid (SEQ ID No: 8) sequences of the RSV G gene and  
5 RSV G protein, respectively;

Figure 8 shows the restriction map of the RSV G gene;

Figure 9 shows the steps involved in the construction of an expression vector containing a  
10 chimeric  $F_{PIV-3}$  -  $F_{RSV}$  gene;

Figure 10 shows the steps involved in the construction of an expression vector containing a  $F_{PIV-3}$  gene lacking the 5'-untranslated sequence and transmembrane anchor and cytoplasmic tail coding regions;

15 Figure 11 shows the steps involved in the construction of an expression vector containing a chimeric  $F_{PIV-3}$  -  $F_{RSV}$  gene containing a truncated PIV-3 F gene devoid of 5'-untranslated region linked to a truncated RSV F1 gene;

20 Figure 12 shows the steps involved in construction of a modified pAC 610 baculovirus expression vector containing a chimeric  $F_{PIV-3}$  -  $F_{RSV}$  gene consisting of the PIV-3 F gene lacking both the 5'-untranslated sequence as well as transmembrane and cytoplasmic tail coding region  
25 linked to the truncated RSV F1 gene;

Figure 13 shows immunoblots of cell lysates from Sf9 cells infected with recombinant baculoviruses;

Figure 14 shows the steps involved in constructing a baculovirus transfer vector (pD2);

30 Figure 15 shows the steps involved in construction of a chimeric  $F_{RSV}$  -  $HN_{PIV-3}$  gene;

Figure 16 shows an SDS-PAGE gel and immunoblot of purified  $F_{RSV}$  -  $HN_{PIV-3}$  chimeric protein;

Figure 17 illustrates mutagenesis of a PIV-3 F gene;

35 and

Figure 18 shows the steps involved in the construction of a chimeric  $F_{PIV-3} - G_{RSV}$  gene.

GENERAL DESCRIPTION OF INVENTION

5 In the present invention, a chimeric molecule protective against two different major childhood diseases is provided. The present invention specifically relates to the formulation of various recombinant Parainfluenza virus (PIV)/Respiratory syncytial virus (RSV) immunogens 10 to produce safe and efficacious vaccines capable of protecting infants and young children, as well as other susceptible individuals, against diseases caused by infection with both PIV and RSV. However, as described above, the present invention extends to the construction 15 of multimeric hybrid genes containing genes coding for protective antigens from many pathogens. Such vaccines may be administered in any desired manner, such as a readily-injectable vaccine, intranasally or orally.

In the present invention, the inventors have 20 specifically engineered several model PIV/RSV chimeric genes containing relevant sequences from selected genes coding for PIV-3 and RSV surface glycoproteins linked in tandem. All genes in the chimeric constructs described herein were obtained from recent clinical isolates of 25 PIV-3 and RSV. The chimeric gene constructs may include gene sequences from either PIV-3 F or HN genes linked in tandem to either RSV F or G genes in all possible relative orientations and combinations.

The chimeric gene constructs provided herein may 30 consist of either the entire gene sequences or gene segments coding for immunogenic and protective epitopes thereof. The natural nucleotide sequence of these genes may be modified by mutation while retaining antigenicity and such modifications may include the removal of 35 putative pre-transcriptional terminators to optimize their expression in eukaryotic cells. The genes were

designed to code for hybrid PIV-RSV surface glycoproteins linked in tandem in a single construct to produce gene products which elicit protective antibodies against both parainfluenza and respiratory syncytial viruses. Such  
5 multimeric hybrid genes consist of a gene sequence coding for a human PIV-3 F or HN protein or an immunogenic epitope-containing fragment thereof linked to a gene sequence coding for a human RSV G or F protein or an immunogenic epitope-containing fragment thereof.  
10 Specific gene constructs which may be employed include  $F_{PIV-3} - F_{RSV}$ ,  $F_{RSV} - HN_{PIV-3}$  and  $F_{PIV-3} - G_{RSV}$  hybrid genes.  
In addition, the present invention also extends to the construction of other multimeric genes, such as trimeric genes containing PIV and RSV genes or gene  
15 segments, linked in all possible relative orientations.  
For example:

$F_{PIV} - HN_{PIV} - F$  or  $G_{RSV}$

$F_{PIV} - F_{RSV} - G_{RSV}$

$HN_{PIV} - F_{RSV} - G_{RSV}$

20 The multimeric genes provided herein also may comprise at least one gene encoding at least one immunogenic and/or immunostimulating molecule.

The multimeric hybrid genes provided herein may be sub-cloned into appropriate vectors for expression in  
25 cellular expression systems. Such cellular expression systems may include bacterial, mammalian, insect and fungal, such as yeast, cells.

The chimeric proteins provided herein also may be presented to the immune system by the use of a live vector, including live viral vectors, such as recombinant  
30 poxviruses, adenoviruses, retroviruses, Semliki Forest viruses, and live bacterial vectors, such as *Salmonella* and mycobacteria (e.g. BCG).

Chimeric proteins, such as a PIV/RSV chimera,  
35 present in either the supernatants or cell lysates of

transfected, transformed or infected cells then can be purified in any convenient manner.

To evaluate the immunogenicity and protective ability of the chimeric proteins, suitable experimental animals are immunized with either varying doses of the purified chimeric proteins, such as the PIV/RSV chimera, and/or live recombinant vectors as described above. Such chimeric proteins may be presented to the immune system by either the use of physiologically-acceptable vehicles, such as aluminum phosphate, or by the use of delivery systems, such as ISCOMS and liposomes. The chimeras also may be formulated to be capable of eliciting a mucosal response, for example, by conjugation or association with immunotargeting vehicles, such as the cholera toxin B subunit, or by incorporation into microparticles. The vaccines may further comprise means for delivering the multimeric protein specifically to cells of the immune system, such as toxin molecules or antibodies. To further enhance the immunoprotective ability of the chimeric proteins, they may be supplemented with other immunogenic and/or immunostimulating molecules. The chimeric PIV/RSV proteins specifically described herein may be formulated with an adjuvant, such as aluminum phosphate, to produce readily-injectable vaccines for protection against the diseases caused by both PIV-3 and RSV. The chimeric proteins also may be administered intranasally or orally. The chimeric proteins may be used in test kits for diagnosis of infection by PIV-3 and RSV.

The invention is not limited to the preparation of chimeric PIV-3 and RSV proteins, but is applicable to the production of chimeric immunogens composed of either the entire sequences or regions of the immunogenic proteins from at least two pathogens sequentially linked in a single molecule. Chimeric antigens also may be synthesized to contain the immunodominant epitopes of

11

several proteins from different pathogens. These chimeric antigens may be useful as vaccines or as diagnostic reagents.

SEQUENCE IDENTIFICATION

5 Several nucleotide and amino acid sequences are referred to in the disclosure of this application. The following table identifies the sequences and the location of the sequence:

10	<u>SEQ ID No.</u>	<u>Identification</u>	<u>Location</u>
15	1	Nucleotide sequence for PCR-amplified PIV-3 F gene	Fig. 1, Example 1
20	2	Amino acid sequence for PCR-amplified PIV-F protein	Fig. 1, Example 1
25	3	Nucleotide sequence for PIV-3 HN gene	Fig. 3, Example 1
30	4	Amino acid sequence for PIV-3 HN protein	Fig. 3, Example 1
35	5	Nucleotide sequence for RSV F gene	Fig. 5, Example 1
40	6	Amino acid sequence for RSV F protein	Fig. 5, Example 1
45	7	Nucleotide sequence for RSV G gene	Fig. 7, Example 1
50	8	Amino acid sequence for RSV G protein	Fig. 7, Example 1
	9	BsrI - BamHI oligo-nucleotide cassette	Fig. 9, Example 2
	10	BspHI - BamHI oligo-nucleotide cassette	Fig. 9, Example 2
	11	EcoRI - Ppu MI oligo-nucleotide cassette	Fig. 9, Example 2
	12	BrsI - BamHI oligo-nucleotide cassette	Fig. 10, Example 3

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- |    |    |                                                        |                     |
|----|----|--------------------------------------------------------|---------------------|
|    | 13 | EcoRI -Bsr BI oligo-nucleotide cassette                | Fig. 10, Example 3  |
| 5  | 14 | EcoRV - EcoRI oligo-nucleotide cassette                | Fig. 11, Example 5  |
|    | 15 | EcoRV - BamHI oligo-nucleotide cassette                | Fig. 14, Example 8  |
| 10 | 16 | BspHI - BspHI oligo-nucleotide cassette                | Fig. 15, Example 9  |
|    | 17 | Nucleotide sequence for PIV-3 F gene                   | Example 15          |
| 15 | 18 | Mutagenic oligo-nucleotide #2721                       | Fig. 17, Example 15 |
| 20 | 19 | Nucleotide sequence for part of oligo-nucleotide #2721 | Example 15          |
| 25 | 20 | Oligonucleotide probe                                  | Example 15          |

DEPOSIT INFORMATION

Certain plasmid DNAs described and referred to herein have been deposited with the American Type Culture Collection (ATCC) located at Rockville, Maryland, USA, pursuant to the Budapest Treaty and prior to the filing of this application. The deposited purified plasmids will become available to the public upon grant of this U.S. patent application or upon publication of its corresponding European patent application, whichever first occurs. The invention described and claimed herein is not to be limited in scope by the plasmid DNAs of the constructs deposited, since the deposited embodiment is intended only as an illustration of the invention. The following purified plasmids were deposited at the ATCC with the noted accession numbers on December 17, 1992:

<u>Plasmid</u>	<u>Example No.</u>	<u>Accession No.</u>
pAC DR7	5	75387
pD2RF-HN	9	75388
pD2F-G	16	75389

Any equivalent plasmids that can be used to produce equivalent antigens as described in this application are within the scope of the invention.

EXAMPLES

5       The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific Examples. These Examples are described solely for purposes of illustration and are not intended to limit the scope of 10 the invention. Changes in form and substitution of equivalents are contemplated as circumstances may suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitations.

15      Methods for cloning and sequencing the PIV-3 and RSV genes as well as the procedures for sub-cloning the genes into appropriate vectors and expressing the gene constructs in mammalian and insect cells are not explicitly described in this disclosure but are well 20 within the scope of those skilled in the art.

Example 1:

25      This Example outlines the strategy used to clone and sequence the PIV-3 F, HN and RSV F, G genes (from a type A isolate). These genes were used in the construction of the  $F_{PIV-3}$  -  $F_{RSV}$ ,  $F_{RSV}-HN_{PIV-3}$ , and  $F_{PIV-3}-G_{RSV}$  chimeric genes detailed in Examples 2 to 4, 9 and 15, respectively.

30      Two PIV-3 F gene clones initially were obtained by PCR amplification of cDNA derived from viral RNA extracted from a recent clinical isolate of PIV-3. Two other PIV-3 F gene clones as well as the PIV-3 HN, RSV F and RSV G genes were cloned from a cDNA library prepared from mRNA isolated from MRC-5 cells infected with clinical isolates of either PIV-3 or RSV (type A isolate). The PIV-3 F (both PCR amplified and non-PCR amplified), PIV-3 HN, RSV F and RSV G gene clones were 35 sequenced by the dideoxynucleotide chain termination

procedure. Sequencing of both strands of the genes was performed by a combination of manual and automated sequencing.

The nucleotide (SEQ ID No: 1) and amino acid (SEQ ID No: 2) sequences of the PCR amplified PIV-3 F gene and F protein, respectively, are presented in Figure 1 and the restriction map of the gene is shown in Figure 2. Sequence analysis of the 1844 nucleotides of two PCR amplified PIV-3 F gene clones confirmed that the clones were identical. Comparison of the coding sequence of the PCR-amplified PIV-3 F gene clone with that of the published PIV-3 F gene sequence revealed a 2.6% divergence in the coding sequence between the two genes resulting in fourteen amino acid substitutions.

The nucleotide sequence of the non-PCR amplified PIV-3 F gene clone differed from the PCR amplified gene clone in the following manner: the non-PCR amplified clone had ten additional nucleotides (AGGACAAAAG) at the 5' untranslated region of the gene and differed at four positions, 8 (T in PCR-amplified gene to C in non-PCR amplified gene) , 512 (C in PCR-amplified gene to T in non-PCR amplified gene) , 518 (G in PCR-amplified gene to A in non-PCR amplified gene) and 1376 (A in PCR-amplified gene to G in non-PCR amplified gene). These changes resulted in three changes in the amino acid sequence of the F protein encoded by the non-PCR amplified PIV-3 F gene. Serine (position 110), glycine (position 112), and aspartic acid (position 398) in the primary amino acid sequence of the F protein encoded by the PCR amplified PIV-3 F gene was changed to phenylalanine (position 110), glutamic acid (position 112) and glycine (position 398), respectively, in the primary amino acid sequence of the F protein encoded by the PCR amplified clone.

Figure 3 shows the nucleotide (SEQ ID No: 3) and amino acid (SEQ ID No: 4) sequences of the PIV-3 HN gene and protein, respectively and the restriction map of the

gene is presented in Figure 4. Analysis of the 1833 nucleotide sequence from two HN clones confirmed that the sequences were identical. A 4.4% divergence in the coding sequence of the PIV-3 HN gene was noted when the 5 sequence was compared to the published PIV-3 HN coding sequence. This divergence resulted in seventeen amino acid substitutions in the amino acid sequence of the protein encoded by the PIV-3 HN gene.

The nucleotide (SEQ ID No: 5) and amino acid (SEQ ID 10 No: 6) sequences of the RSV F gene and RSV F protein, respectively, are shown in Figure 5 and the restriction map of the gene is shown in Figure 6. Analysis of the 1887 nucleotide sequence from two RSV F clones verified complete sequence homology between the two clones. 15 Comparison of this nucleotide sequence with that reported for the RSV F gene revealed approximately 1.8% divergence in the coding sequence resulting in eleven amino acid substitutions.

The nucleotide (SEQ ID No: 7) and amino acid (SEQ ID 20 No: 8) sequences of the RSV G gene and RSV G protein, respectively, are presented in Figure 7 while the restriction map of the gene is outlined in Figure 8. Comparison of the 920 nucleotide sequence of the G gene 25 clone with the published G sequence (type A isolate) revealed a 4.2% divergence in the nucleotide sequence and a 6.7% divergence in the amino acid sequence of the gene product. This divergence resulted in twenty amino acid substitutions.

The full-length PIV-3 F (non-PCR amplified), PIV-3 30 HN, RSV F and RSV G genes were cloned into λgt11 and subcloned into the multiple cloning site of a Bluescript M13-SK vector, either by blunt end ligation or using appropriate linkers. The PCR-amplified PIV-3 F gene was directly cloned into the Bluescript vector. The cloning 35 vectors containing the PIV-3 F-PCR amplified, PIV-3 F non-PCR amplified, PIV-3 HN, RSV F and RSV G genes were

named pPI3F, pPI3Fc, pPIVHN, pRSVF and pRSVG, respectively.

Example 2:

This Example illustrates the construction of a Bluescript-based expression vector (pMCR20) containing the chimeric F<sub>PIV-3</sub> - F<sub>RSV</sub> gene. This chimeric gene construct contains the 5' untranslated region of the PIV-3 F gene but lacks the hydrophobic anchor and cytoplasmic tail coding regions of both the PIV-3 and RSV F genes. The steps involved in the construction of this plasmid are summarized in Figure 9.

To prepare the PIV-3 portion of the chimeric gene (Figure 9, step 1), the full length PIV-3 gene lacking the transmembrane region and cytoplasmic tail coding regions was retrieved from plasmid pPI3F by cutting the polylinker with BamHI, blunt-ending the linearized plasmid with Klenow polymerase and cutting the gene with BsrI. A BsrI-BamHI oligonucleotide cassette (SEQ ID No: 9) containing a PpuMI site and three successive translational stop codons were ligated to the truncated 1.6 Kb [BamHI]-BsrI PIV-3 F gene fragment and cloned into the EcoRV-BamHI sites of a Bluescript M13-SK expression vector containing the human methallothionen promoter and the poly A and IVS sequences of the SV40 genome (designated pMCR20), to generate plasmid pME1.

To engineer the RSV F gene component of the chimeric construct (Figure 9, step 2), the RSV F gene lacking the transmembrane region and cytoplasmic tail coding regions was retrieved from plasmid pRSVF by cutting the polylinker with EcoRI and the gene with BspHI. A synthetic BspHI-BamHI oligonucleotide cassette (SEQ ID No: 10) containing three successive translational stop codons was ligated to the 1.6 Kb truncated RSV F gene and cloned into the EcoRI-BamHI sites of the Bluescript based expression vector, pMCR20 to produce plasmid pES13A. Plasmid pES13A then was cut with EcoRI and PpuMI to

remove the leader and F2 coding sequences from the truncated RSV F gene. The leader sequence was reconstructed using an EcoRI-PpuMI oligocassette (SEQ ID No: 11) and ligated to the RSV F1 gene segment to generate plasmid pES23A.

To prepare the chimeric  $F_{PIV-3}-F_{RSV}$  gene (Figure 9, step 3) containing the 5' untranslated region of the PIV-3 F gene linked to the truncated RSV F1 gene fragment, plasmid pME1 (containing the 1.6 Kb truncated PIV-3 F gene) first was cut with PpuMI and BamHI. The PpuMI-BamHI restricted pME1 vector was dephosphorylated with intestinal alkaline phosphatase. The 1.1 Kb RSV F1 gene fragment was retrieved from plasmid pES23A by cutting the plasmid with PpuMI and BamHI. The 1.1 Kb PpuMI-BamHI RSV F1 gene fragment was cloned into the PpuMI-BamHI sites of the dephosphorylated pME1 vector to generate plasmid pES29A. This chimeric gene construct contains the 5' untranslated region of the PIV-3 F gene but lacks the nucleotide sequences coding for the hydrophobic anchor domains and cytoplasmic tails of both the PIV-3 and RSV F proteins.

Example 3:

This Example illustrates the construction of a Bluescript-based expression vector containing the PIV-3 F gene lacking both the 5' untranslated and transmembrane anchor and cytoplasmic tail coding regions. The steps involved in constructing this plasmid are outlined in Figure 10.

Plasmid pPI3F containing the full length PIV-3 F gene was cut with BamHI, blunt ended with Klenow polymerase and then cut with Bsrl to remove the transmembrane and cytoplasmic tail coding regions. The Bluescript-based expression vector, pMCR20, was cut with SmaI and BamHI. A synthetic Bsrl-BamHI oligonucleotide cassette (SEQ ID No: 12) containing a translational stop codon was ligated with the 1.6 Kb blunt ended-Bsrl PIV-3

F gene fragment to the SmaI-BamHI restricted pMCR20 vector to produce plasmid pMpFB. The PIV-3 F gene of this construct lacked the DNA fragment coding for the transmembrane and cytoplasmic anchor domains but 5 contained the 5' untranslated region. To engineer a plasmid containing the PIV-3 F gene devoid of both the 5' untranslated region and the DNA fragment coding for the hydrophobic anchor domain, plasmid pMpFB was cut with EcoRI and BstBI. An EcoRI-BstBI oligocassette (SEQ ID 10 No: 13) containing the sequences to reconstruct the signal peptide and coding sequences removed by the EcoRI-BstBI cut was ligated to the EcoRI-BstBI restricted pMpFB vector to produce plasmid pMpFA.

Example 4:

15 This Example illustrates the construction of the chimeric  $F_{PIV-3}-F_{RSV}$  gene composed of the truncated PIV-3 F gene devoid of the 5' untranslated region linked to the truncated RSV F1 gene. The steps involved in constructing this plasmid are summarized in Figure 11.

20 To prepare this chimeric gene construct, plasmid pES29A (Example 2) was cut with BstBI and BamHI to release the 2.5 Kb BstBI-BamHI PI3-3 F-RSV F1 chimeric gene fragment. This BstBI-BamHI fragment was isolated from a low melting point agarose gel and cloned into the 25 BstBI-BamHI sites of the dephosphorylated vector pMpFA to produce plasmid pES60A. This construct contained the PIV-3 F gene lacking both the 5' untranslated region and the hydrophobic anchor and cytoplasmic tail coding sequences linked to the F1 coding region of the truncated 30 RSV F gene. This chimeric gene was subsequently subcloned into the baculovirus transfer vector (see Example 5).

Example 5:

35 This Example illustrates the construction of the modified pAC 610 baculovirus transfer vector containing the native polyhedrin promoter and the chimeric  $F_{PIV-3}-F_{RSV}$

gene consisting of the PIV-3 F gene lacking both the 5' untranslated sequence and the nucleotide sequence coding for the hydrophobic anchor domain and cytoplasmic tail linked to the truncated RSV F1 gene. Construction of 5 this plasmid is illustrated in Figure 12.

The pAC 610 baculovirus expression vector was modified to contain the native polyhedrin promoter in the following manner. Vector pAC 610 was cut with EcoRV and BamHI. The 9.4 Kb baculovirus transfer vector lacking 10 the EcoRV-BamHI DNA sequence was isolated from a low melting point agarose gel and treated with intestinal alkaline phosphatase. In a 3-way ligation, an EcoRV-EcoRI oligonucleotide cassette (SEQ ID No: 14) containing the nucleotides required to restore the native polyhedrin 15 promoter was ligated with the 1.6 Kb EcoRI-BamHI truncated RSV F gene fragment isolated from construct pES13A (Example 2, step 2) and the EcoRV-BamHI restricted pAC 610 phosphatased vector to generate plasmid pES47A. To prepare the pAC 610 based expression vector containing 20 the chimeric  $F_{PIV-3}$ - $F_{RSV}$  gene, plasmid pES47A was first cut with EcoRI and BamHI to remove the 1.6 Kb truncated RSV F gene insert. The 2.8 Kb  $F_{PIV-3}$ - $F_{RSV}$  chimeric gene was retrieved by cutting plasmid pES60A (Example 4) with 25 EcoRI and BamHI. The 2.8 Kb EcoRI-BamHI chimeric gene was ligated to the EcoRI-BamHI restricted pES47A vector to generate plasmid pAC DR7 (ATCC 75387).

Example 6

This Example outlines the preparation of plaque-purified recombinant baculoviruses containing the 30 chimeric  $F_{PIV-3}$  -  $F_{RSV}$  gene.

*Spodoptera frugiperda* (Sf9) cells were co-transfected with 1.0  $\mu$ g wild-type AcMNPV DNA and 2.5  $\mu$ g of  $F_{PIV-3}$ - $F_{RSV}$  plasmid DNA (plasmid pAC DR7 - Example 5). Putative recombinant baculoviruses (purified once by 35 serial dilution) containing the  $F_{PIV-3}$  -  $F_{RSV}$  chimeric gene were identified by dot-blot hybridization. Lysates of

insect cells infected with the putative recombinant baculoviruses were probed with the  $^{32}\text{P}$ -labelled  $\text{F}_{\text{PIV-3}}-\text{F}_{\text{RSV}}$  chimeric gene insert. Recombinant baculoviruses were plaque-purified twice before being used for expression studies. All procedures were carried out according to the protocols outlined by M.D. Summers and G.E. Smith in "A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures", Texas Agricultural Experiment Station, Bulletin 1555, 1987.

10     Example 7:

This Example illustrates the presence of the chimeric  $\text{F}_{\text{PIV-3}}-\text{F}_{\text{RSV}}$  protein in supernatants and cell lysates of infected Sf9 cells.

Insect cells were infected with the plaque-purified recombinant baculoviruses prepared as described in Example 6 at a m.o.i. of 8. Concentrated supernatants from cells infected with the recombinant viruses were positive in a PIV-3 F specific ELISA. In addition, when lysates from  $^{35}\text{S}$ -methionine labelled infected cells were subjected to SDS-polyacrylamide gel electrophoresis and gels were analyzed by autoradiography, a strong band with apparent molecular weight of approximately 90 kDa was present in lysates of cells infected with the recombinant viruses but was absent in the lysates from wild-type infected cells. The presence of the chimeric  $\text{F}_{\text{PIV-3}}-\text{F}_{\text{RSV}}$  protein in the lysates of cells infected with the recombinant baculoviruses was confirmed further by Western blot analysis using monospecific anti-PIV-3 F and anti-RSV F antisera and/or monoclonal antibodies (Mabs). Lysates from cells infected with the recombinant baculoviruses reacted with both anti-PIV-3 and anti-RSV antisera in immunoblots. As shown in the immunoblot of Figure 13, lysates from cells infected with either the RSV F or  $\text{F}_{\text{PIV-3}}-\text{F}_{\text{RSV}}$  recombinant baculoviruses reacted positively with the anti-F RSV Mab. As expected, lysates from cells infected with wild type virus did not react.

with this Mab. In addition, only lysates from cells infected with the chimeric F<sub>PIV-3</sub> - F<sub>RSV</sub> recombinant viruses reacted with the anti-PIV-3 F<sub>1</sub> antiserum.

Example 8

5 This Example illustrates modification of the baculovirus transfer vector pVL1392 (obtained from Invitrogen), wherein the polyhedrin ATG start codon was converted to ATT and the sequence CCG was present downstream of the polyhedrin gene at positions +4,5,6.  
10 Insertion of a structural gene several base pairs downstream from the ATT codon is known to enhance translation. The steps involved in constructing this modified baculovirus transfer vector are outlined in Figure 14.

15 The baculovirus expression vector pVL1392 was cut with EcoRV and BamHI. The 9.5 kb restricted pVL1392 vector was ligated to an EcoRV-BamHI oligonucleotide cassette (SEQ ID No: 15) to produce the pD2 vector.

Example 9:

20 This Example illustrates the construction of the pD2 baculovirus expression vector containing the chimeric F<sub>RSV</sub> -HN<sub>PIV-3</sub> gene consisting of the truncated RSV F and PIV-3 HN genes linked in tandem. The steps involved in constructing this plasmid are summarized in Figure 15.

25 To engineer the F<sub>RSV</sub>-HN<sub>PIV-3</sub> gene, the RSV F gene lacking the nucleotide sequence coding for the transmembrane domain and cytoplasmic tail of the RSV F glycoprotein was retrieved from plasmid pRSVF (Example 1) by cutting the polylinker with EcoRI and the gene with BspHI. The PIV-3 HN gene devoid of the DNA fragment coding for the hydrophobic anchor domain was retrieved from plasmid pPIVHN (Example 1) by cutting the gene with BspHI and the polylinker with BamHI. The 1.6 Kb EcoRI-BspHI RSV F gene fragment and the 1.7 Kb BspHI-BamHI PIV-3 HN gene fragment were isolated from low melting point agarose gels. For cloning purposes, the two BspHI sites

in the Bluescript based mammalian cell expression vector, pMCR20, were mutated. Mutations were introduced in the BspHI sites of the pMCR20 by cutting the expression vector with BspHI, treating both the BspHI restricted vector and the 1.1 Kb fragment released by the BspHI cut with Klenow polymerase and ligating the blunt-ended 1.1 Kb fragment to the blunt-ended Bluescript-based expression vector to generate plasmid pM'. Since insertion of the 1.1 Kb blunt-end fragment in the mammalian cell expression vector in the improper orientation would alter the Amp<sup>r</sup> gene of the Bluescript-based expression vector, only colonies of HB101 cells transformed with the pM' plasmid DNA with the 1.1 Kb blunt-ended fragment in the proper orientation could survive in the presence of ampicillin. Plasmid DNA was purified from ampicillin-resistant colonies of HB101 cells transformed with plasmid PM' by equilibrium centrifugation in cesium chloride-ethidium bromide gradients. The 1.6 Kb EcoRI-BspHI RSV F and 1.7 Kb BspHI-BamHI PIV-3 HN gene fragments were directly cloned into the EcoRI-BamHI sites of vector pM' in a 3-way ligation to generate plasmid pM' RF-HN.

To restore specific coding sequences of the RSV F and PIV-3 HN genes removed by the BspHI cut, a BspHI-BspHI oligonucleotide cassette (SEQ ID No: 16) containing the pertinent RSV F and PIV-3 HN gene sequences was ligated via the BspHI site to the BspHI-restricted plasmid pM' RF-HN to produce plasmid pM RF-HN. Clones containing the BspHI-BspHI oligonucleotide cassette in the proper orientation were identified by sequence analysis of the oligonucleotide linker and its flanking regions.

To clone the chimeric F<sub>RSV</sub>-HN<sub>PIV-3</sub> gene into the baculovirus expression vector pD2 (Example 8), the F<sub>RSV</sub>-HN<sub>PIV-3</sub> truncated gene first was retrieved from plasmid pM RF-HN by cutting the plasmid with EcoRI. The 3.3 Kb F<sub>RSV</sub>-

HN<sub>PIV-3</sub> gene then was cloned into the EcoRI site of the baculovirus transfer vector plasmid pD2 to generate plasmid pD2 RF-HN (ATCC 75388). Proper orientation of the 3.3 Kb EcoRI F<sub>RSV</sub>-HN<sub>PIV-3</sub> chimeric gene insert in plasmid pD2 RF-HN was confirmed by sequence analysis.

Example 10:

This Example outlines the preparation of plaque-purified recombinant baculoviruses containing the chimeric F<sub>RSV</sub>-HN<sub>PIV-3</sub> gene.

10 Spodoptera frugiperda (Sf9) cells were co-transfected with 1 µg wild-type AcNPV DNA and 2 µg of F<sub>RSV</sub>-HN<sub>PIV-3</sub> plasmid DNA (plasmid pD2 RF-HN-Example 9). Putative recombinant baculoviruses (purified once by serial dilution) containing the F<sub>RSV</sub>-HN<sub>PIV-3</sub> chimeric gene 15 were identified by dot-blot hybridization. Lysates of insect cells infected with the putative recombinant baculoviruses were probed with the <sup>32</sup>P-labelled RSV F or PTV-3 HN gene oligonucleotide probes. Recombinant baculoviruses were plaque-purified three times before 20 being used for expression studies. All procedures were carried out according to the protocols outlined by Summers and Smith (Example 6).

Example 11:

This Example illustrates the presence of the 25 chimeric F<sub>RSV</sub>-HN<sub>PIV-3</sub> protein in supernatants of infected Sf9 and High 5 cells.

Insect cells (Sf9 and High 5), maintained in serum free medium EX401, were infected with the plaque purified recombinant baculoviruses of Example 10 at a m.o.i. of 5 30 to 10 pfu/cell. Supernatants from cells infected with the recombinant baculoviruses tested positive for expressed protein in both the RSV-F and PIV-3 HN specific ELISAS. In addition, supernatants from infected cells reacted positively with both an anti-F RSV monoclonal 35 antibody and anti-HN peptide antisera on immunoblots. A distinct band of approximately 105 kDa was present in the

immunoblots. These results confirm the secretion of the chimeric F<sub>RSV</sub>-HN<sub>PIV-3</sub> protein into the supernatant of Sf9 and High 5 cells infected with the recombinant baculoviruses.

5   Example 12:

This Example illustrates the purification of the chimeric F<sub>RSV</sub>-HN<sub>PIV-3</sub> protein from the supernatants of infected High 5 cells.

High 5 cells, maintained in serum free medium, were 10 infected with the plaque purified recombinant baculoviruses of Example 10 at a m.o.i of 5 pfu/cell. The supernatant from virus infected cells was harvested 2 days post-infection. The soluble F<sub>RSV</sub>-HN<sub>PIV-3</sub> chimeric protein was purified from the supernatants of infected 15 cells by immunoaffinity chromatography using an anti-HN PIV-3 monoclonal antibody. The anti-HN monoclonal antibody was coupled to CNBr-activated Sepharose 4B by conventional techniques. The immunoaffinity column was washed with 10 bed volumes of washing buffer (10mM Tris-HCl pH 7.5, 150 mM NaCl, 0.02% v/v Triton-X 100) prior to 20 use. After sample loading, the column was washed with 10 bed volumes of washing buffer followed by 3 bed volumes of high salt buffer (10mm Tris-HCl pH 7.5, 500mM NaCl, 0.02% v/v Triton-X 100). The chimeric F<sub>RSV</sub>-HN<sub>PIV-3</sub> protein 25 was eluted from the immunoaffinity column with 100 MM glycine, pH 2.5, in the presence of 0.02% Triton X-100. Eluted protein was neutralized immediately with 1M Tris-HCl, pH 10.7.

Polyacrylamide gel electrophoretic analysis (Fig. 30 16, panel A) of the immunoaffinity-purified F<sub>RSV</sub>-HN<sub>PIV-3</sub> protein revealed the presence of one major protein band with an apparent molecular weight of 105 kDa. The purified protein reacted with both an anti-RSV F monoclonal antibody and anti-HN peptide antisera on 35 immunoblots (Fig. 16, panel B, lanes 1 and 2, respectively).

Example 13:

This Example illustrates the immunogenicity of the F<sub>RSV</sub>-HN<sub>PIV-3</sub> protein in guinea pigs.

Groups of four guinea pigs were injected  
5 intramuscularly with either 1.0 or 10.0 µg of the  
chimeric F<sub>RSV</sub>-HN<sub>PIV-3</sub> protein purified as described in  
Example 12 and adjuvanted with aluminum phosphate.  
Groups of control animals were immunized with either  
placebo, or live PIV-3 or RSV (administered  
10 intranasally). Guinea pigs were bled 2 and 4 weeks after  
the primary injection and boosted at 4 weeks with an  
equivalent dose of the antigen formulation. Serum  
samples also were taken 2 and 4 weeks after the booster  
dose. To assess the ability of the chimeric protein to  
15 elicit PIV-3 and RSV-specific antibody responses, sera  
samples were analyzed for the presence of PIV-3 specific  
hemagglutination inhibiting and neutralizing antibodies  
as well as RSV neutralizing antibodies. As summarized in  
Table 1 below (the Tables appear at the end of the  
20 disclosure), the sera of animals immunized with two 10 µg  
doses of the chimeric protein had titres of PIV-3  
specific hemagglutination inhibition (HAI) and PIV-3/RSV  
neutralizing antibodies at the 6 and 8 week time points  
which were equivalent to the levels obtained following  
25 intranasal inoculation with either live PIV-3 or RSV. In  
addition, animals immunized with only two 1 µg doses of  
the chimeric protein elicited strong PIV-3 and RSV  
specific neutralizing antibodies. These results  
confirmed the immunogenicity of both the RSV and PIV-3  
30 components of the chimeric protein and provided  
confirmatory evidence that a single recombinant immunogen  
can elicit neutralizing antibodies against both RSV and  
PIV-3.

Example 14:

This Example illustrates the immunogenicity and protective ability of the F<sub>RSV</sub>-HN<sub>PIV-3</sub> protein in cotton rats.

Groups of eight cotton rats were injected 5 intramuscularly with either 1.0 or 10.0 ug of the chimeric F<sub>RSV</sub>-HN<sub>PIV-3</sub> protein (prepared as described in Example 12) adjuvanted with aluminum phosphate. Groups of control animals were immunized with either placebo (PBS + aluminum phosphate) or live PIV-3 or RSV 10 (administered intranasally). Cotton rats were bled 4 weeks after the primary injection and boosted at 4 weeks with an equivalent dose of the antigen formulation. Serum samples were also taken 1 week after the booster dose. As shown in Table 2 below, data from the 4-week 15 bleed demonstrated that both a 1 and 10 µg dose of the chimeric protein was capable of inducing a strong primary response. Reciprocal mean log<sub>2</sub> PIV-3 specific HAI and PIV-3/RSV neutralizing titers were equivalent to the titres obtained with live PIV-3 and RSV. Thus, a single 20 inoculation of the chimeric protein was sufficient to elicit neutralizing antibodies against both PIV-3 and RSV. Strong neutralizing PIV-3 and RSV titres also were observed following the booster dose (5 week bleed). These results provide additional evidence that both the 25 RSV and PIV-3 components of the chimeric protein are highly immunogenic.

To assess the ability of the chimeric immunogen to simultaneously protect animals against both RSV and PIV-3, four cotton rats from each group were challenged 30 intranasally with 100 TCID<sub>50</sub> units of either PIV-3 or RSV. Animals were killed 4 days after virus challenge. Virus titers were determined in lung homogenates. As shown in Table 3 below, animals immunized with either 1 or 10 µg of the chimeric F<sub>RSV</sub>-HN<sub>PIV-3</sub> protein were completely 35 protected against challenge with either PIV-3 or RSV. These results provide evidence that the chimeric protein

is not only highly immunogenic but can also simultaneously protect cotton rats against disease caused by both PIV-3 and RSV infection.

Example 15:

5        This Example illustrates the construction of a Bluescript M13-SK vector containing the chimeric  $F_{PIV-3}$ - $G_{RSV}$  gene. This chimeric gene construct contains the 5' untranslated region of a mutated PIV-3 F gene but lacks the nucleotide sequence coding for the hydrophobic anchor  
10      and cytoplasmic tail domains of both a mutated PIV-3 F and the native RSV G genes. The steps involved in constructing this plasmid are outlined in Figures 17 and 18.

The first step (Fig. 17) involved in preparing the  
15      PIV-3 F component of the chimeric  $F_{PIV-3}$ - $G_{RSV}$  gene construct was to eliminate the putative pre-termination sites within the 18 nucleotide long sequence 5' CAAGAAAAAGGAATAAAA 3' (SEQ ID No: 17) located between positions 857 and 874 of the non PCR-amplified PIV-3 F  
20      gene and positions 847 and 864 of the PCR-amplified PIV-3 F gene (see Figure 1). To this end, the PIV-F cDNA of the non-PCR amplified PIV-3 F gene was cut at the BsaAI and EcoRI sites. The BsaAI-EcoRI PIV F gene fragment was cloned into the EcoRI site of a Bluescript M13-SK vector  
25      using an EcoRI-BsaAI linker. The 857-874 target region of the PIV-3 F gene (non-PCR amplified) then was mutated by oligonucleotide-mediated mutagenesis using the method of Morinaga et al. [1984, Biotechnology 2: 636-639]. Plasmid pPI3Fc (Example 1) was cut with ScaI in the Amp' gene and dephosphorylated with alkaline phosphatase (plasmid #1). A second sample of plasmid pPI3Fc was cut with BstEII and NsiI to produce a 3.9 Kb restricted plasmid, lacking the 0.9 Kb BstEII-NsiI fragment of the PIV-3 F gene (plasmid #2). A mutagenic 78-mer synthetic  
30      oligonucleotide (#2721 shown in Fig. 17-SEQ ID No: 18)) containing the sequence 5' CAGGAGAAGGGTATCAAG 3' (SEQ ID  
35      No: 19) was annealed to the 3' end of the BstEII-NsiI fragment of plasmid #2 and used to transform E. coli JM109.

No: 19) was synthesized to specifically mutate the 857-874 DNA segment without changing the F protein sequence. This oligonucleotide was added to plasmid DNAs #1 and #2, denatured at 100°C for 3 min. and renatured by gradual cooling. The mixture then was incubated in the presence of DNA polymerase, dNTPs and T4 ligase and transformed into HB101 cells. Bacteria containing the 1.8 Kb mutated PIV-3 F gene were isolated on YT agar plates containing 100 µg/ml ampicillin. Hybridization with the 10 oligonucleotide probe 5' AGGAGAAGGGTATCAAG 3' (SEQ ID No: 20) was used to confirm the presence of the mutated PIV-3 F gene. The mutated gene sequence was confirmed by DNA sequencing. The plasmid containing the mutated PIV-3 gene was designated pPI3Fm.

The second step (Fig. 18) in the engineering of the chimeric gene construct involved constructing a Bluescript based vector to contain the truncated PIV-3 Fm gene lacking the nucleotide sequence coding for the transmembrane anchor domain and cytoplasmic tail of the 15 PIV-3 F protein linked in tandem with the RSV G gene lacking both the 5' leader sequence and the nucleotide sequence coding for the transmembrane anchor domain and cytoplasmic tail of the G glycoprotein.

To prepare this chimeric gene, the orientation of 20 the mutated PIV-F gene in plasmid pPI3Fm first was reversed by EcoRI digestion and religation to generate plasmid pPI3Fmr. To prepare the PIV-3 F gene component of the chimeric gene, plasmid pPI3Fmr was cut with NotI and BsrI to release the 1.7 Kb truncated PIV-3 F gene. 25 To prepare the RSV G component, the 0.95 Kb RSV-G gene lacking both the 5' leader sequence and the DNA segment encoding the G protein anchor domain and cytoplasmic tail was released from plasmid pRSVG (Example 1) by cutting the polylinker with EcoRI and the gene with BamHI. The 30 0.95 Kb EcoRI-BamHI RSV G gene fragment was subcloned into the EcoRI-BamHI sites of a restricted Bluescript 35

vector, pM13-SK, to produce plasmid pRSVGt. The 0.95 Kb EcoRI-BamHI G gene fragment and the 1.5 Kb NotI-BsrI truncated PIV-3 F gene were linked via a BsrI-BamHI oligonucleotide cassette (SEQ ID No: 9) restoring the F and G gene coding sequences and cloned into the pRSVGt vector restricted with BamHI and NotI in a 3-way ligation. The plasmid thus generated was designated pFG.

Example 16:

This Example outlines the construction of the pD2 baculovirus transfer vector (described in Example 8) containing the chimeric  $F_{PIV-3}-G_{RSV}$  gene consisting of a mutated PIV-3 F gene lacking the hydrophobic anchor and cytoplasmic coding regions linked to the RSV G gene lacking both the 5' leader sequence and the nucleotide sequences encoding the transmembrane anchor domain and cytoplasmic tail of the G protein.

To prepare this construct, plasmid pFG (Example 15) was cut with EcoRI to release the 2.6 Kb  $F_{PIV-3}-G_{RSV}$  chimeric gene. The 2.6 Kb EcoRI restricted chimeric gene fragment then was sub-cloned into the EcoRI site of the dephosphorylated pD2 vector to generate the 12.1 Kb plasmid pD2F-G (ATCC 75389).

Example 17:

This Example outlines the preparation of plaque-purified recombinant baculoviruses containing the chimeric  $F_{PIV-3}-G_{RSV}$  gene.

Spodoptera frugiperda (Sf9) cells were co-transfected with 2 ug of pD2F-G plasmid DNA (Example 16) and 1 ug of linear wild-type AcNPV DNA (obtained from Invitrogen). Recombinant baculoviruses containing the  $F_{PIV-3}-G_{RSV}$  gene were plaque-purified twice according to the procedure outlined in Example 10.

Example 18:

This Example illustrates the presence of the chimeric  $F_{PIV-3}-G_{RSV}$  protein in the supernatant of Sf9 and High 5 cells infected with the recombinant baculoviruses.

Sf9 and High 5 cells were infected with recombinant baculoviruses containing the F<sub>PIV-3</sub>-G<sub>RSV</sub> gene (Example 16) at a m.o.i. of 5 to 10 pfu/cell. The supernatant of cells infected with the recombinant viruses tested positive for expressed protein in the PIV-3 F specific ELISA. Supernatants of infected cells reacted with both anti-F PIV-3 and anti-G RSV monoclonal antibodies in immunoblots. These results confirm the presence of the chimeric F<sub>PIV-3</sub>-G<sub>RSV</sub> protein in the supernatants of infected Sf9 and High 5 cells.

Example 19:

This Example outlines the preparation of recombinant vaccinia viruses expressing the F<sub>PIV-3</sub> - F<sub>RSV</sub> and F<sub>RSV</sub> - HN<sub>PIV-3</sub> genes.

Vaccinia virus recombinant viruses expressing the F<sub>PIV-3</sub>-F<sub>RSV</sub> (designated vP1192) and F<sub>RSV</sub>-HN<sub>PIV-3</sub> (designated vP1195) genes were produced at Virogenetics Corporation (Troy, NY) (an entity related to assignee hereof) using the COPAK host-range selection system. Insertion plasmids used in the COPAK host-range selection system contained the vaccinia K1L host-range gene [Perkus et al., (1990) *Virology* 179:276-286] and the modified vaccinia H6 promoter [Perkus et al. (1989), *J. Virology* 63:3829-3836]. In these insertion plasmids, the K1L gene, H6 promoter and polylinker region are situated between Copenhagen strain vaccinia flanking arms replacing the ATI region [open reading frames (ORFs) A25L, A26L; Goebel et al., (1990), *Virology* 179: 247-266; 517-563]. COPAK insertion plasmids are designed for use in in vivo recombination using the rescue virus NYVAC (vP866) (Tartaglia et al., (1992) *Virology* 188: 217-232). Selection of recombinant viruses was done on rabbit kidney cells.

Recombinant viruses, vP1192 and vP1195 were generated using insertion plasmids pES229A-6 and PSD.RN, respectively. To prepare plasmid pES229A-6 containing

the  $F_{PIV.3}-F_{RSV}$  gene, the COPAK-H6 insertion plasmid pSD555 was cut with SmaI and dephosphorylated with intestinal alkaline phosphatase. The 2.6 Kb  $F_{PIV.3}-F_{RSV}$  gene was retrieved from plasmid pES60A (Example 4) by cutting the 5 plasmid with EcoRI and BamHI. The 2.6 Kb EcoRI-BamHI  $F_{PIV.3}-F_{RSV}$  gene was blunt ended with Klenow polymerase, isolated from a low melting point agarose gel and cloned into the SmaI site of the COPAK-H6 insertion plasmid pSD555 to generate plasmid pES229A-6. This positioned 10 the  $F_{PIV.3}-F_{RSV}$  ORF such that the 5' end is nearest the H6 promoter.

To prepare plasmid PSD.RN, the pSD555 vector first was cut with SmaI and BamHI. Plasmid pM RF-HN (Example 9) containing the truncated  $F_{RSV}-HN_{PIV.3}$  gene was cut with 15 ClaI, blunt ended with Klenow polymerase and then cut with BamHI. The 3.3 Kb  $F_{RSV}-HN_{PIV.3}$  gene was cloned into the SmaI-BamHI sites of the pSD555 vector to generate plasmid PSD.RN. This positioned the  $F_{RSV}-HN_{PIV.3}$  ORF such that the H6 5' end is nearest the H6 promoter.

20 Plasmids pES229A-6 and PSD.RN were used in in vitro recombination experiments in vero cells with NYVAC (vP866) as the rescuing virus. Recombinant progeny virus was selected on rabbit kidney (RK)-13 cells (ATCC #CCL37). Several plaques were passaged two times 25 on RK-13 cells. Virus containing the chimeric genes were confirmed by standard *in situ* plaque hybridization [Piccini et al. (1987), Methods in Enzymology, 153:545-563] using radiolabeled probes specific for the PIV and RSV inserted DNA sequences. Plaque purified virus 30 containing the  $F_{PIV.3}-F_{RSV}$  and  $F_{RSV}-HN_{PIV.3}$  chimeric genes were designated vP1192 and vP1195, respectively.

Radioimmunoprecipitation was done to confirm the expression of the chimeric genes in vP1192 and vP1195 infected cells. These assays were performed with lysates 35 prepared from infected Vero cells [according to the procedure of Taylor et al., (1990) J. Virology 64, 1441-

1450] using guinea pig monospecific PIV-3 anti-HN and  
anti-F antiserum and rabbit anti-RSV F antiserum. Both  
the anti-PIV F and anti-RSV F antisera precipitated a  
protein with an apparent molecular weight of  
5 approximately 90 kDa from vP1192 infected Vero cells.  
Both anti-RSV F and guinea pig anti-PIV HN antisera  
precipitated a protein with an apparent molecular weight  
of approximately 100 kDa from vP1195 infected cells.  
These results confirmed the production of the  $F_{PIV-3}-F_{RSV}$   
10 and  $F_{RSV}-HN_{PIV-3}$  chimeric proteins in Vero cells infected  
with the recombinant poxviruses.

SUMMARY OF DISCLOSURE

In summary of the disclosure, the present invention  
provides multimeric hybrid genes which produce chimeric  
15 proteins capable of eliciting protection against  
infection by a plurality of pathogens, particularly PIV  
and RSV. Modifications are possible within the scope of  
this invention.

**Table 1 Secondary antibody response of guinea pigs immunized with the chimeric f<sub>RSV</sub>-HNPIV-3 protein**

Antigen Formulation	Dose (ug)	HAI Titre <sup>a</sup> ( $\log_2 \pm$ s.e.)		Neutralization Titre <sup>b</sup> ( $\log_2 \pm$ s.e.)			
		PIV-3	RSV	PIV-3	RSV	PIV-3	RSV
		6 wk Bleed	8 wk Bleed	6 wk Bleed	8 wk Bleed	6 wk Bleed	8 wk Bleed
Buffer	-	<1.0 ± 0.0	<1.0 ± 0.0	<1.0 ± 0.0	<1.0 ± 0.0	<1.0 ± 0.0	<1.0 ± 0.0
f <sub>RSV</sub> -HNPIV-3	10.0	9.1 ± 0.3	9.1 ± 0.3	7.1 ± 0.3	7.1 ± 0.5	5.5 ± 0.9	4.5 ± 1.2
	1.0	7.0 ± 2.0	7.3 ± 2.2	5.0 ± 1.5	4.5 ± 1.4	4.5 ± 0.5	3.0 ± 1.0
Live PIV-3		8.6 ± 0.7	7.3 ± 0.6	7.0 ± 0.4	7.3 ± 0.6	N/A	N/A
Live RSV		N/A <sup>c</sup>	N/A	N/A	N/A	5.5 ± 1.5	5.0 ± 1.0

<sup>a</sup> Reciprocal mean  $\log_2$  serum dilution which inhibits erythrocyte agglutination by 4 hemagglutinating units of PIV-3

<sup>b</sup> Reciprocal mean  $\log_2$  serum dilution which blocks hemadsorption of 100 TCID<sub>50</sub> units of PIV-3 or RSV

<sup>c</sup> N/A - not applicable

Table 2: Serum antibody response of cotton rats immunized with the chimeric F<sub>RSV</sub>-H<sub>PIV-3</sub> protein<sup>a</sup>

Antigen Formulation	Dose (ug)	HAI Titre <sup>b</sup> ( $\log_2 \pm$ s.d.)		Neutralization Titre <sup>c</sup> ( $\log_2 \pm$ s.d.)			
		PIV-3	RSV	4 wk Bleed	5 wk Bleed	4 wk Bleed	5 wk Bleed
Buffer	-	2.8 ± 0.5	<3.0 ± 0.0	<1.0 ± 1.0	<1.0 ± 0.0	1.8 ± 0.3	0.8 ± 0.7
F <sub>RSV</sub> -H <sub>PIV-3</sub>	10.0	9.5 ± 1.3	10.5 ± 0.6	>9.0 ± 0.0	>9.0 ± 0.0	5.2 ± 1.1	5.8 ± 0.9
	1.0	9.3 ± 1.0	10.3 ± 0.5	>9.0 ± 0.0	>9.0 ± 0.0	5.0 ± 0.7	5.8 ± 1.2
Live PIV-3		7.0 ± 0.0	8.5 ± 0.7	>9.0 ± 0.0	9.2 ± 0.7	N/A	N/A
Live RSV		N/A <sup>d</sup>	N/A	N/A	N/A	5.5 ± 0.6	8.5 ± 0.6

<sup>a</sup> Each value represents the mean titre of antisera from 8 animals.

<sup>b</sup> Reciprocal mean log<sub>2</sub> serum dilution which inhibits erythrocyte agglutination by 4 hemagglutinating units of PIV-3

<sup>c</sup> Reciprocal mean log<sub>2</sub> serum dilution which blocks hemadsorption of 100 TCID<sub>50</sub> units of PIV-3 or RSV

<sup>d</sup> N/A - not applicable

Table 3. Response of immunized cotton rats to PIV/RSV challenge<sup>a</sup>

Antigen Formulation	Dose (ug)	Mean virus lung titre $\log_{10}/g$ lung $\pm$ s.d.	
		RSV	PIV-3
Buffer	-	$3.7 \pm 0.3$	$3.4 \pm 0.3$
$F_{RSV-HN_{PIV-3}}$	10.0	$\leq 1.5 \pm 0.0$	$\leq 1.5 \pm 0.0$
$F_{RSV-HN_{PIV-3}}$	1.0	$\leq 1.5 \pm 0.0$	$\leq 1.5 \pm 0.0$
Live RSV		$\leq 1.5 \pm 0.0$	$\leq 1.5 \pm 0.0$
Live PIV-3		$\leq 1.5 \pm 0.0$	$\leq 1.5 \pm 0.0$

<sup>a</sup> Animals were challenged intranasally with 100 TCID<sub>50</sub> units of PIV-3 or RSV and killed 4 days later. Each value represents the mean virus lung titre of 4 animals.

CLAIMS

What we claim is:

1. A multimeric hybrid gene, comprising a gene sequence coding for an antigenic region of a protein from a first pathogen linked to a gene sequence coding for an antigenic region of a protein from a second pathogen.
2. The hybrid gene of claim 1 wherein said first and second pathogens are selected from bacterial and viral pathogens.
3. The hybrid gene of claim 2 wherein both said first and second pathogens are viral pathogens.
4. The hybrid gene of claim 1 wherein said first and second pathogens are selected from those causing different respiratory tract diseases.
5. The hybrid gene of claim 4 wherein said first and second pathogens causing different respiratory tract diseases are selected from the paramoxyviridae family of viruses.
6. The hybrid gene of claim 1 wherein at least one of said gene sequences is mutated while retaining antigenicity.
7. The hybrid gene of claim 6 wherein said mutation is at a putative pre-termination site.
8. The hybrid gene of claim 1 wherein said first pathogen is parainfluenza virus (PIV) and said second pathogen is respiratory syncytial virus (RSV).
9. The hybrid gene of claim 1, comprising at least one gene sequence coding for a parainfluenza virus (PIV) protein linked to at least one gene sequence coding for a respiratory syncytial virus (RSV) protein.
10. The hybrid gene of claim 9, wherein said parainfluenza virus protein is selected from PIV-3 F and HN proteins and said respiratory syncytial virus protein is selected from RSV G and F proteins.
11. The hybrid gene of claim 1 consisting of a gene sequence coding for a human PIV-3 F or HN protein or an

immunogenic epitope-containing fragment thereof linked to a gene sequence coding for a human RSV G or F protein or an immunogenic epitope-containing fragment thereof.

12. The hybrid gene of claim 11 which is selected from  $F_{PIV-3} - F_{RSV}$ ,  $F_{RSV} - HN_{PIV-3}$  and  $F_{PIV-3} - G_{RSV}$  hybrid genes.

13. The hybrid gene of claim 1 contained in an expression vector.

14. The hybrid gene of claim 13 in the form of plasmid pAC DR7, pD2 RF-HN or pD2 F-G.

15. The hybrid gene of claim 1 further comprising at least one gene encoding at least one immunogenic and/or immunostimulating molecule.

16. Cells containing the multimeric hybrid gene of claim 1 for expression of a chimeric protein encoded by said gene.

17. The cells of claim 16 which are bacterial cells, mammalian cells, insect cells, yeast cells or fungal cells.

18. A chimeric protein, comprising an antigenic region of a protein from a first pathogen linked to an antigenic region of a protein from a second pathogen.

19. The protein of claim 18, wherein said first and second pathogens are selected from bacterial and viral pathogens.

20. The protein of claim 19 wherein both said first and second pathogens are viral pathogens.

21. The protein of claim 18, wherein said first and second pathogens are selected from those causing different respiratory tract diseases.

22. The protein of claim 21 wherein said first and second pathogens causing different respiratory tract diseases are selected from the paramoxyviridae family of viruses.

23. The protein of claim 18, wherein said first pathogen is parainfluenza virus (PIV) and said second pathogen is respiratory syncytial virus (RSV).

24. The protein of claim 18 comprising at least one parainfluenza virus (PIV) protein linked to at least one respiratory syncytial virus (RSV) protein.

25. The protein of claim 24, wherein said PIV protein is selected from PIV-3 F and HN proteins and said RSV protein is selected from RSV G and F proteins.

26. The protein of claim 18 consisting of a human parainfluenza virus-3 (PIV-3) F or HN protein or an immunogenic epitope-containing fragment thereof linked to a human respiratory syncytial virus (RSV) G or F protein or an immunogenic epitope-containing fragment thereof.

27. The protein of claim 26 which is selected from  $F_{PIV-3} - F_{RSV}$ ,  $F_{RSV} - HN_{PIV-3}$  and  $F_{PIV-3} - G_{RSV}$  chimeric proteins.

28. A process for preparation of a chimeric protein which comprises:

isolating a gene sequence coding for an antigenic region of a protein from a first pathogen,

isolating a gene sequence coding for an antigenic region of a protein from a second pathogen,

linking said gene sequences to form a multimeric hybrid gene, and expressing the multimeric hybrid gene in a cellular expression system

29. The process of claim 28 wherein said multimeric hybrid gene comprises a gene sequence coding for a PIV-F or HN protein or an immunogenic epitope-containing fragment thereof linked to a gene sequence coding for a human RSV G or F protein or an epitope-containing fragment thereof.

30. The process of claim 29 wherein said multimeric hybrid gene is selected from  $F_{PIV-3} - F_{RSV}$ ,  $F_{RSV} - HN_{PIV-3}$  and  $F_{PIV-3} - G_{RSV}$  hybrid genes.

31. The process of claim 30 wherein said multimeric hybrid gene is contained in an expression vector comprising plasmid pAC QR7, pD2 RF-HN or pD2 F-G.

32. The process of claim 28 wherein said cellular expression system is provided by bacterial cells,

mammalian cells, insect cells, yeast cells or fungal cells.

33. The process of claim 32 including separating a chimeric protein from a culture of said cellular expression system and purifying the separated chimeric protein.

34. A live vector for antigen delivery containing the gene of claim 1.

35. The live vector of claim 34 which is a viral vector.

36. The live vector of claim 35 wherein said viral vector is selected from poxviral, adenoviral and retroviral viral vectors.

37. The live vector of claim 34 which is a bacterial vector.

38. The live vector of claim 37 wherein said bacterial vector is selected from salmonella and mycobacteria.

39. A vaccine against diseases caused by multiple pathogenic infections, comprising a chimeric protein comprising an antigen region of a protein from a first pathogen linked to an antigenic region of a protein from a second pathogen, and a physiologically-acceptable carrier therefor.

40. The vaccine of claim 39, wherein said first and second pathogens are selected from bacterial and viral pathogens.

41. The vaccine of claim 39, which also contains at least one other immunogenic and/or immunostimulating molecule.

42. The vaccine of claim 40 wherein both said first and second pathogens are viral pathogens.

43. The vaccine of claim 39, wherein said first and second pathogens are selected from those causing upper and lower respiratory tract diseases.

44. The vaccine of claim 39, wherein said first pathogen is parainfluenza virus (PIV) and said second pathogen is respiratory syncytial virus (RSV).

45. The vaccine of claim 39 against infection by both parainfluenza virus (PIV) and respiratory syncytial virus (RSV), comprising a recombinant multimeric protein containing at least one segment consisting of a PIV protein or an immunogenic epitope-containing fragment thereof linked to at least one segment consisting of a RSV protein or an immunogenic epitope-containing fragment thereof, and a carrier therefor.

46. The vaccine of claim 45 wherein said recombinant multimeric protein is a recombinant chimeric protein containing a segment consisting of a PIV-3 F or HN protein or an immunogenic epitope-containing fragment thereof linked to a segment consisting of an RSV G or F protein or an immunogenic epitope-containing fragment thereof.

47. The vaccine of claim 46 containing at least one additional protein of PIV or RSV or chimeric protein thereof.

48. The vaccine of claim 39 wherein said carrier comprises an adjuvant.

49. The vaccine of claim 39 wherein said carrier is an ISCOM, a liposome or a microparticle.

50. The vaccine of claim 46 formulated to be administered in an injectable form, intranasally or orally.

51. The vaccine of claim 39 further comprising means for delivering said multimeric protein specifically to cells of the immune system.

52. The vaccine of claim 51 wherein said delivery means comprises a toxin molecule or an antibody.

53. A vaccine against diseases caused by multiple pathogenic infection, comprising a live vector as claimed in claim 34, and a physiologically-acceptable carrier therefor.

54. A method of immunizing a host against diseases caused by multiple pathogenic infections, which comprises

administering to a host an effective amount of a vaccine as claimed in claim 28 or 53.

55. The method of claim 54 wherein said vaccine is against diseases caused by parainfluenza virus (PIV) and respiratory syncytial virus (RSV).

56. The method of claim 55 wherein said host is selected from infants, young children, pregnant women, women of child-bearing age and susceptible persons.

57. A diagnostic reagent for detecting infection by a plurality of different pathogens in a host, comprising the chimeric protein claimed in claim 18.

58. A method of detecting infection by a plurality of different pathogens in a host, which comprises using said chimeric protein claimed in claim 18.

**FIG.1A.** NUCLEOTIDE SEQUENCE OF THE PIV-3 F GENE (PCR-AMPLIFIED)

AAGTCAATAACCAACTATTAGCAGTCATACTGGAAACAGAACAGAAAGAAAGAGATCAA  
TTCAAGTTATGGTTGATAATCGTCAAGTATGGTACGTCTTGTTCTCTTAAGTT  
10 20 30 40 50 60

AAAAGCTAAATAAGAGAAATCAAAACAAAGGTATAAGAACACCCGAAACAAACAAATCAAA  
TTTCGATTATCTCTTTAGTTTGGCTTATCTTGTGGCTTGTGGCTTGTGGCTTGTGGCTT  
70 80 90 100 110 120

CATCCAACTTAAACAAATTC  
 GTAGGTTAGGTAAATTGTTAAGGTTCTGGCGTTCTGTTGTTG  
 130 140 150 160 170 180  
 MET PRO THR [LEU] ILE LEU ILE THR THR MET ILE MET ALA [SER] SER CYS GLN  
 ACAATGCCAACTTAATACTGCTTAATTACAAATGATTATGGCATTCTGC  
 TGTACGGTTGAAATTGACGATTAAATGTTACAAATACCGTAGAAGGACGGTT  
 190 200 210 220 230 240  
 SP

ILE ASP ILE THR LYS LEU GLN HIS VAL GLY VAL LEU VAL ASN SER PRO LYS GLY MET LYS  
 ATACATATCACAAACTACAGGCACTAGGTAGGTATTGGTCAACAGTCCCCAAAGGGATGAAG  
 TATGTTAGTGTGTTGATGTACATCCACATAACCAGGTTTCTCTACTTC  
 250 260 270 280 290 300

ILE SER GLN ASN PHE GLU THR ARG TYR LEU ILE LEU SER LEU ILE PRO LYS ILE GLU ASP	
ATATCACAAACCTTCGGAAACAAAGATATCTAATTGAGCCTCATACCCAAAATAAGAAC	
TATAGTGTGAAAGCTTTCTATAGATTAAAACCTGGAGTATGGTTATCTTCTG	
310	330
320	340
	350
	360

SER ASN SER CYS GLY ASP GLN GLN ILE LYS GLN TYR LYS ARG LEU LEU ASP ARG LEU ILE  
TCATAACTCTTGTGGT GACCAACAGATCAAACAAATACAAGAGGTTATGGATAAGACTGATC  
AATTGAAACACCCTGGTTGCTAGTTATGTTACCTATCTGACTAG  
370 380 390 400 410 420

**FIG.1B.** ILE PRO LEU TYR ASP GLY LEU ARG LEU GLN LYS ASP VAL ILE VAL **[THR]** ASN GLN GLU SER  
 ATCCCTTATGATGGATTAAAGATTACAGAAAGATGTGATAAGTAAACCAATCAAGAAC  
 TAGGAGATACTACCTAATTCTAATGTTCTACACTATCATGGTTAGTTAG  
 430 440 450 460 470 480

F2-F1 CLEAVAGE SITE

ASN GLU ASN THR ASP PRO ARG THR **[ARG]** ARG **[SER]** PHE GLY GLY VAL ILE GLY THR ILE ALA  
 ATGAAAACACTGATCCCAGAACAAAGACGATCCTTGAGGGTAAATTGGAAACCATTC  
 TTACTTTGACTAGGGTCTGCTTAGGAAACCTCCGCATTAACCTGGTAACGA  
 490 500 510 520 530 540

LEU GLY VAL ALA THR SER ALA GLN ILE THR ALA ALA VAL ALA LEU VAL GLU ALA LYS GLN  
 CTGGGAGTAGCAACCTCACGCCATTACAGCCGGCAGTTGCTCTGTTGAAAGCCAG  
 GACCCCTCATCGTTGGAGTTGGAGCTTCTGGTTAGCTTGGTTAGTCC  
 550 560 570 580 590 600

ALA **[LYS]** SER ASP ILE GLU LYS LEU LYS GLU ALA ILE ARG ASP THR ASN LYS ALA VAL GLN  
 GCAAAATCACACATCGAAAAACTCAAAGAACGAAATCACAGGGACACAAACAAAGCAG  
 CGTTTAGTGTAGCTTGGAGTTGGAGCTTCTGGTTAGTCC  
 610 620 630 640 650 660

SER VAL GLN SER SER ILE GLY ASN LEU ILE VAL ALA ILE LYS SER VAL GLN ASP TYR VAL  
 TCAGTTCAAGAGCTCTAGGAAATTAGGAAATTAGGAAATTAGGAAATTAGGAA  
 AGTCAAGCTCGAGATATCCCTTAATTATCATCGTTAATTAGTCAAGGTTCTAAACAG  
 670 680 690 700 710 720

ASN **[ASN]** GLU ILE VAL PRO SER ILE ALA ARG LEU GLY CY<sub>5</sub> GLU ALA ALA GLY LEU GLN LEU  
 AACAAAGGAATATCGGTGCCATGGCTGGATTAGGACTTACGATGGCTGAACTCAATT  
 TTGTTGGCTTACGGTAGCTAACGATCTGATCCAAACACTTCGTTGAAAGTTAA  
 730 740 750 760 770 780

GLY ILE ALA LEU THR GLN HIS TYR SER GLU LEU THR ASN ILE PHE GLY ASP ASN ILE GLY  
 GGAAATTGGCATTAAACACAGCATTACTCAAGAATTAAACACATTTGGTGAATACAGGA  
 CCTTAAACGTTAATGGTGTGGTAAATGAGTCCTTATGTTGTTGTTACCATTTGATCT  
 790 800 810 820 830 840

**FIG. IC.** SER LEU GLN GLU LYS GLY ILE LYS LEU GLN GLY ILE ALA SER LEU TYR ARG THR ASN ILE  
 TCGTTACAAGAAAAAGGAATAATTACAAAGGTATAGCATCATTATACCCACAAATAC  
 AGCAATGTTCTTCTTAAATTAAATTGTTCCATATCGTAGTAATATGGCGTTTATAAG  
 850 870 880 890 900

THR GLU ILE PHE THR THR SER THR VAL ASP LYS TYR ASP ILE TYR ASP LEU LEU PHE THR  
 ACAGAAATATTCAACACATCAACAGTTGATAAAATGATATCTATGATCTATTATTACA  
 TGTCTTATAAGTTGTTGAGCTAACATTAACTATAGATAACTAGATAATAATGATG  
 910 930 940 950 960

GLU SER ILE LYS VAL ARG VAL ILE ASP VAL ASP LEU ASN ASP TYR SER ILE THR LEU GLN  
 GAAATCAAAGGTGAGATTAGATTTGATTGATTTGAAATGATTACTCAATCACCCCAA  
 CTAGTATTTCACCTCAATCTACAAACTAAACTAAACTAAACTAAACTAAACTAA  
 970 980 990 1000

VAL ARG LEU PRO LEU LEU THR ARG LEU LEU ASN THR GLN ILE TYR **[LYS]** VAL ASP SER ILE  
 GTCAAGACTCCCTTAACTAGGCTTGTGAACACTCAAGATCTACAAAGTAGATTCCATA  
 CAGTCTGAGGGAATAATTGATECCGACCACTTGTGAGCTAGATGTTCAAGGTAT  
 1030 1040 1050 1060 1070

SER TYR ASN ILE GLN ASN ARG GLU TRP TYR ILE PRO LEU PRO SER HIS ILE MET THR LYS  
 TCATATAATACTCCAAAACAGAGAAATGGTATATCCCTCTCCAGCCATATCATGAGAAA  
 AGTATAATTAGTTTGTCTTACCATATGGAGAGAAAGGGTATAAGTACTGCTT  
 1090 1100 1110 1120 1130

GLY ALA PKE LEU GLY ALA ASP VAL LYS GLU CYS ILE GLU ALA PHE SER SER TYR ILE  
 GGGCATTCTAGTTGGAGCAGATGTCAAGGAATGTATAGAACCTTCAAGCAGITATA  
 CCCGTAAGATCCACCTCGTCTACAGTTACATATCTCGTAAGTCGGTCAATATA  
 1150 1160 1170 1180 1190

CYS PRO SER ASP PRO GLY PHE VAL LEU ASN HIS GLU KET GLU SER CYS LEU SER GLY ASN  
 TGCCTCTGATCCAGGATTGTACTAACCAACCAGAAATGGAGAGCTTATCAGGAAAC  
 ACGGAAGACTAGGTCTAACATGATTGGTACTTACCTCTCGACAAATAGTCTTG  
 1220 1230 1240 1250 1260

3/39



5/39

FIG. I

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6/39

## RESTRICTION MAP OF THE PIV-3 F GENE

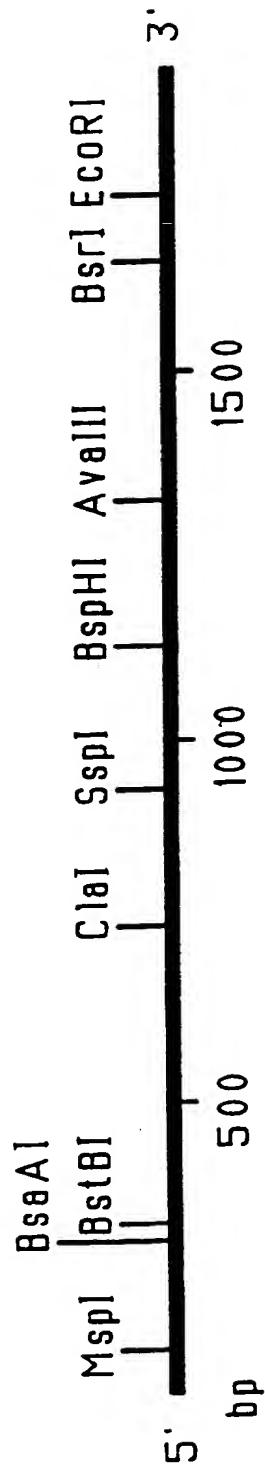


FIG.2.

### FIG. 3A: NUCLEOTIDE SEQUENCE OF THE PIV-3 HN GENE



SRE CYS SER LEU ALA LEU LEU ASN THR ASP VAL TYR GLN LEU CYS SER THR PRO LYS VAL  
 GTCA TGT CCT GCA CCT AAATACAGA TGTATAACTGTTCAACTGTTCAACTCCAAAGT GAGGGTTCA  
 CAGTACAAGAGATCGTGAGGATTATGTCTACATAGTGAACAAAGT GAGGGTTCA  
 840  
 800 820 840

ASP GLU ARG SER ASP TYR ALA SER SER GLY ILE GLU ASP ILE VAL LEU ASP ILE VAL ASN  
 TAGT GAAAAGATCAGATTAGCATCAGGCCATAGAAAGATAATTGACTTACITGATTTGTC  
 ACTACCTTCTAGTCTTAATACGGTAGTACGTAGTACATGAACTAACATGAACTAACAGT  
 850  
 860 870 880 890

**[TYR]** ASP GLY SER ILE SER THR THR ARG PHE LYS ASN ASN ILE SER PHE ASP GLN PRO  
 TTATGATGGCTCAATCTCAACAAACAAGATTAAAGAAATAACATAAGCTTGGATCAACC  
 AATACATACCGAGATTAGAGTTAGTTCTAAATTCTTATTATGTATTGTTGG  
 910  
 920 930 940 950

TYR ALA ALA LEU TYR PRO SER VAL GLY PRO GLY ILE TYR TYR LYS GLY LYS ILE ILE PHE  
 TTATGCTGCACTTACCCATCTGGACCAAGGATATACTACAAAGGGAAATAAAATT  
 AATACGACGTGATATGGGTAGACAACTGGTCCCTATGATGTTCCGTTATTAA  
 970  
 980 990 1000 1010

LEU GLY TYR GLY LEU GLU HIS PRO ILE ASN GLU ASN **[VAL]** ILE CYS ASN THR THR GLY  
 TCTCGGGTATGGAGGTCTTGAACATCCAATTAATGAGAATGTTACTCTACCTTACCTTACATTAGACGT  
 AAGGCCATACCTCCAGAACCTGGCTTGTGTTGGTT  
 1030  
 1040 1050 1060 1070

CYS PRO GLY LYS THR GLN ARG ASP CYS ASN GLN ALA SER HIS SER PRO TRP PHE SER ASP  
 GTGTCGGGGAAACACAGAGAGACTGCATGGCATCTCATAGTCCATGGTTGTTGAGA  
 CACAGGGCCCTTGTGTTCTGAGTTAGCTGAGTACGGTACGGTACGGTACGGTACGGT  
 1090  
 1100 1110 1120 1130

ARG ARG MET VAL ASN SER ILE ILE VAL VAL ASP LYS GLY LEU ASN SER ILE PRO LYS LEU  
 TAGGAGGATGGTCAACTCTATCATTTGTTGACAAAGGGCTTAAACTCAATTCCAAATT  
 ATCCCTACCAAGTTGAGATAGTAACAAACAAACTGTTCCGAAATTGAGTTAAGGGTTTAA  
 1150  
 1160 1170 1180 1190

FIG.3C.

LYS VAL TRP THR ILE SER MET ARG GLN ASN TYR TRP GLY SER GLU GLY ARG LEU LEU  
 645 GAGGTATGGACGATACTTATGAGACAGAAATTACTGGGGTCAAGAAGGAGTTACCTCT  
 660 CTTCCATACCTGCTATAGATACTCTTATGACCCCAGTCCTCCATGAGA  
 675 1220 1230 1240 1250 1260

THR GLY VAL TYR THR ASP ALA TYR PRO LEU ASN PRO THR GLY SER ILE VAL SER SER VAL  
 AACAGGAGTATATACTGATGCATACTCCACTCAATCCACAGGGAGCATTTGTCTATCTGT  
 TTGTCCTCATATGACTACGTTAGGTGAATTAGGTAACTGGTACAGTAGACA  
 1450 1460 1470 1480 1490 1490

ILE LEU ASP SER GLN LYS SER ARG VAL ASN PRO VAL ILE THR TYR SER THR [ALA] THR GLU  
CAT ATT GAT TCA CAA AAA TCG GAG AGT GAA ACC CAG T CATA CT ACT CAA ACAG GAA ACC GGA  
GT TAA TCA AAT GT TTA GCT CACT TGG GTC AGT GAT TGA AT GAG TT GGT TGG CT  
1510 1520 1530 1540 1550 1560

ARG VAL ASN GLU LEU ALA ILE	<b>[Arg]</b>	ASN ARG THR LEU SER ALA GLY TYR THR THR SER
AAG AGT TAA AC GAG CCT GGC AT CCA GAA AC ACT CT CAG GCT GGA TATA CAA ACA AAG		
TTC CAT TGG CT GAC CGG TAG CC TTT GGT AGT GAG TGT GAC CT AT GAT TGT GTT GTC		
1570	1580	1590
		1600
		1610
		1620

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FIG. 3D.

CYS ILE THR HIS TYR ASN LYS GLY TYR CYS PHE HIS ILE VAL GLU ILE ASN [GLN] LYS SER  
 CTGGCATCACACTATAACAAAGGATATTCTATAGAAATAGAAATACAGAAAAG  
 GACGTAGTGTGATATTGTTCCATAACAAAGTATACTCTTATAGCTTTC  
 1630 1640 1650 1660 1670 1680

AATCAGACAA TAGACAAA AGGGAAATAAAAA  
TTAGTCTTATCTGTTATCTTCCCTTATTATT  
1830  
1820  
1810

NUCLEOTIDE SEQUENCE OF THE PIV-3 HN GENE. THE CDNA SEQUENCE IS SHOWN IN THE PLUS (mRNA) STRAND SENSE IN THE 5' TO 3' DIRECTION. THE TRANSMEMBRANE (TM) ANCHOR DOMAIN IS UNDERLINED. AMINO ACIDS DIFFERING FROM THE PUBLISHED PRIMARY SEQUENCE OF THE PROTEIN ENCODED BY THE PIV-3 HN GENE ARE BOXED.

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12/39

## RESTRICTION MAP OF THE PIV-3 HN GENE

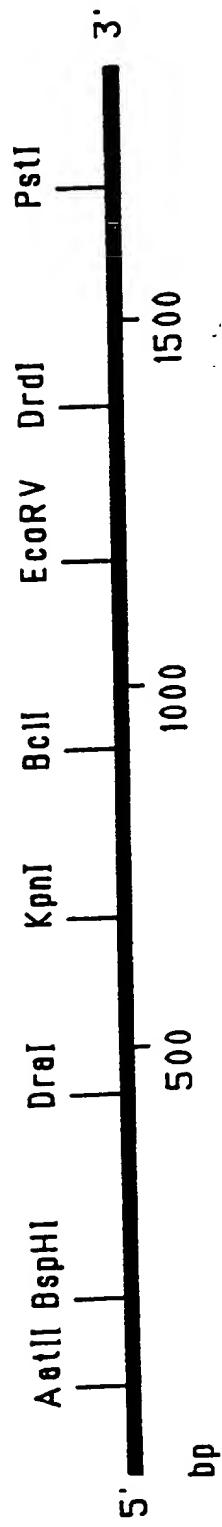


FIG. 4.

**FIG. 5A.** NUCLEOTIDE SEQUENCE OF THE RSV F GENE.

5' MET GLU LEU [PRO] ILE LEU LYS ALA ASN ALA ILE THR THR ILE LEU ALA [ALA] VAL THR PHE  
 ATGGAGTTGCAATCCTCAAAAGCAAATGCCATTACCAATCCCTGCAGTCACATT  
 TACCTCAACGGGTAGGTTACGGTACGGTAAATGGTTAGGAGCTCAGTGTAAA  
 10 20 30 40 50 60

CYS PHE [ASN] SER GLN ASN ILE THR GLU GLU PHE TYR GLN SER THR CYS SER ALA VAL  
 TGCCTTGCTTCTAGTCAGTCAAACATCAGTTGACTTAAATAGTTAGTGTACCT  
 ACCAAACGAAAGATCAGTTGACTTAAATAGTTAGTGTACCT  
 70 80 90 100 110 120

SER LYS GLY TYR LEU SER ALA LEU ARG THR GLY TRP TYR THR SER VAL ILE THR ILE GLU  
 AGCAAAAGGCTTACCTTACCTGCTTAAGGAACTAGCTAAAGAACCTGGTTACCT  
 TCGGTTCCGATAGAAATCACCGAGATTGACCAACCATACT  
 130 140 150 160 170 180

LEU SER ASN ILE LYS GLU ASN LYS CYS ASN GLY THR ASP ALA LYS VAL LYS LEU [MET] LYS  
 TTAAAGTAATATCAAGGAAATAAGTTAATGGAAACAGATGCTAAGGTTAAATTGATAAA  
 ATTCAATTATAGTTCTTACCTTACATTACCT  
 190 200 210 220 230 240

GLN GLU LEU ASP LYS TYR LYS ASN ALA VAL THR GLU LEU MET GLN SER THR  
 CAAGAATTAGATAATAATAAAATGCTGTAACAGAAATTGAGTTGCTCATGCAAGCACA  
 GTTCTTAATCTTACGACATTGTTACGTTACGTTACGTTACGTTACGTTACGTT  
 250 260 270 280 290 300

PRO [ALA] [ALA] ASN ASN ARG ALA ARG ARG GLU LEU PRO ARG PHE MET ASN TYR THR LEU ASN  
 CCAGCAGCAACATCGAGCCAGAGAAACTACCAAGGTTATGAAATTACACTAAC  
 GGTCTGCTCTGTTAGCTCTGCTTACCTTAATGTTACCTTAATGTTGAGTTG  
 310 320 330 340 350 360

ASN [THR] LYS LYS THR ASN VAL THR LEU SER LYS ARG LYS ARG ARG PHE LEU GLY PHE  
 AAT ACC CAA AAA ACC AAAT GTC ATTAAG CAAG GAA AGA AGA ATTTCT TGGTTT  
 TTATG GTTT GGT TAC ATTTG GTT ACAT TGT AATT CGT CCT TCT CTT  
 390 400 410 420

LEU LEU GLY VAL GLY SER ALA ILE ALA SER GLY [ILE] ALA VAL SER LYS VAL LEU HIS LEU  
 TTGTTAGGTGTTGGATCTGCATAATGCCAATGCCAGTGGCATGCTGTATCTAAGGTCCTGCACCTTA  
 AACAAATCCACAAACCTAGCCTAGAACGTTAGGATGATAAGGTTGTTGGTCAAGGACATAGCTA  
 430 440 450 460

GLU GLY GLU VAL ASN LYS ILE LYS SER ALA LEU SER THR ASN LYS ALA VAL VAL SER  
 GAAGGAGAAAGTGAACAAAGATCACAAAGCTACTATCCACAAACAAAGGCCGCTAGTCAG  
 CTTCCCTTCACCTGGTTAGTTCAAGGAAATCGAAT  
 490 500 510 520

LEU SER ASN GLY VAL SER VAL LEU THR SER LYS VAL LEU ASP LEU LYS ASN TYR ILE ASP  
 TTATCAAATGGAGTTAGTGTCTTAAACGCAAGTGGCTTACCTAAACAAACTATAGAT  
 AATAGTTACCTCAATCAGAAATGGTGGTTGAGTTGAGTT  
 550 560 570 580

LYS GLN LEU LEU PRO ILE VAL ASN LYS ARG SER CYS [ARG] ILE SER ASN ILE GLU THR VAL  
 AAACAAATTGTTACCTATTGAAATTAAGCGAAGCTGCGAGAAATACAAATAGAAACTGTC  
 TTGTTAACAAATGGATAACACTTTCGCTTGCTGACGTCTAAGTT  
 610 620 630 640

ILE GLU PHE GLN HIS LYS ASN ASN ARG LEU LEU GLU ILE THR ARG GLU PHE SER VAL LEU  
 ATAGAGTTCCAAACACAAACAGACTAGAGATTACAGGGAAATTAGTTAGTT  
 TATCTCAAGGGTTGTTGTTCTGTT  
 670 680 690 700

ALA GLY VAL THR THR PRO VAL SER THR TYR MET LEU THR ASN SER GLU LEU SER LEU  
 GCAGGGTTAACTACACCTGTTAAAGCAACTATAGTTAACTAACTGTT  
 CGTCACATGGTGGACATTCGTTGAAAT  
 730 740 750 760

**FIG.5B.**

ILE ASN ASP MET PRO ILE THR ASN ASP GLN LYS LEU MET SER ASN ASN VAL GLN ILE  
 ATCAATGATAATGCCATAACAAATGATCAGAAAAAGTTAATGTCACAAATGTTCAATTACAGTTGTTACAAGTTAT  
 TAGTTACTATACGGATAATTGCTTACTAGTTACGGAATT  
 790 800 810 820 830 840

VAL ARG GLN GLN SER TYR SER ILE MET SER ILE ILE LYS GLU GLU VAL LEU ALA TYR VAL  
 GTTAGACGAAAGT TACCTCATGTCATGAGATGATAGTACAGGTATTATTTCTCCATTGAAATC GTATACAT  
 CAACTGTCTGGT 850  
 860  
 870  
 880  
 890  
 900

VAL GLN LEU PRO LEU TYR GLY VAL ILE ASP THR PRO CYS TRP LYS LEU HIS THR SER PRO  
 910  
 G T A C A A T T A C C A C T A T G G T G A T A G A C C T T G G A A A T T A C A C A C A T C C C C T  
 920  
 C A T G T T A T G G T G A T A C C A C T A T G G A A C A C T T A A T G T G T G A G G G A  
 930  
 940  
 950  
 960

LEU CYS THR ASN THR LYS GLU GLY SER ASN ILE CYS LEU THR ARG THR ASP ARG GLY  
 CTATGACAACCAACAAAGAAGGGTCAAACATCTGTAAACAAGAACTGACAGAGGA  
 GATACATGTTGGTTGTTCTCCAGTTGACAAATTGTTGACTGTCCTTGTCTCTT  
 970 990 1000 1020

LEU CYS ASN VAL ASP ILE PHE ASN PRO LYS TYR ASP CYS ILE MET THR SER LYS THR  
 CTC TGC CAAT GTT GAC AT TCA AT CCC AAAT TGA TT GAT TTT GAT TAA AT GCT AAT CAA AAAC A  
 GAG CCT TA CAA CT GT TAA G T A G G G T T A C T A A C T T A A C T G A A G T T G A T T G T G  
 1150 1160 1170 1180 1190 1200

ASP VAL SER SER SER VAL ILE THR SER LEU GLY ALA ILE VAL SER CYS TYR GLY LYS THR  
 GAT GAA CGG TCC GGT ATC ACG ATC TCA GAT CAC ATG GAG ATC GGT CCA AAC  
 CT ACG ATT CTC GAG GCA ATG TAG AGT CCG TGG AAG CAG TAC GAT ACC GTT GA  
 1230 1240 1250 1260

LYS CYS THR ALA SER ASN LYS ASN ARG GLY ILE LYS THR PHE SER ASN GLY CYS ASP  
 AA AT GAT CAG GCAT CCA AT TAA AA AT C G T G G A A T C A T A A A G A C A T T C T A A C G G C A A C T  
 TT A C A T G T C G T A G G T T A T T A G C A C C T A G T T A G T C A T T G T A A A G A T T G C C C A C T A  
 1270 1280 1290 1300 1310 1320

TYR VAL SER ASN LYS GLY **VAL** ASP THR VAL SER VAL GLY ASN THR LEU TYR TYR VAL ASN  
 T A T G T A T C A A A T A A G G G G T G G A C A C T G T G T G T A G G T A A C A C A T T A T T A T G T A A A T  
 A T A C A T A G T T A T T C C C C A C C T G A C A G A C A T C C A T G T G T A A T A A C A T T A  
 1330 1340 1350 1360 1370 1380

LYS GLN GLU GLY LYS SER LEU TYR VAL LYS GLY GLU PRO ILE ILE ASN PHE TYR ASP PRO  
 A A G C A A G G C A A A G G T G A A C C A T T A A G G T G A A C C A A T A A T T C T A G C C C A  
 T T C G T T C T C C G T T T C A G A G A T A C A T T T C A C C T G T T A T T A A G A T A C T G G G T  
 1390 1400 1410 1420 1430 1440

LEU VAL PHE PRO SER ASP GLU PHE ASP ALA SER ILE SER GLN VAL ASN GLU LYS ILE ASN  
 T T A G T A T T C C C T G T G A A T T C A G T C A A G T C A A G T C A A G A G A T T A A C  
 A A T C A T A A G G G A G A C T T A A C T T A A C T T A A G T T C A G T T G C T C T C A A T T  
 1450 1460 1470 1480 1490 1500

GLN SER LEU ALA PHE ILE ARG LYS SER ASP GLU LEU LEU HIS ASN VAL ASN ALA GLY LYS  
 C A G A G T T A G G C A T T A T T C G T T A A T C G A T G A A T T A C A T T A G T A A T G C T C A A T  
 G T C T C A A T C G T A A T A A G C A T T A G G C T A C T T A A T G T T A A T G T T A C T T A C  
 1510 1520 1530 1540 1550 1560

SER THR THR ASN ILE MET ILE THR THR ILE ILE GLU ILE ILE VAL ILE LEU LEU SER  
 T C A A C C A A A T A T C A T G A A T A A C T A A T C A T G A A T T A G A G A T T A T G T T A C A  
 A G T T G G T T A T T A G T A C T A T C T C A A T A T C T C A A T A C A A T A C A A T A C  
 1570 1580 1590 1600 1610 1620

TM

**FIG. 5D.**

→ ←

LEU	ILE	ALA	VAL	GLY	LEU	LEU	TYR	CYS	LYS	ALA	ARG	SER	THR	PRO	VAL	THR	LEU	SER	
TAA	TAT	GCT	GTT	GACT	GCT	CTT	ACT	GTA	AAG	GCC	AGA	ACG	CAC	ACT	AAG	CAC	GGT	GAT	TG
ATT	ACG	ACA	ACT	GAC	ACT	GGT	ACAT	GAC	ATG	CCG	GGT	GGT	GTG	ATG	GGT	GGT	GGT	GTG	ATG
1630	1640	1650	1660	1670	1680														
LYS, ASP	GLN	LEU	SER	GLY	ILE	ASN	ASN	ILE	ALA	PHE	SER	ASN							
AGG	GAT	CAACT	GAG	TGG	TAA	TAA	TAA	TAT	TGC	CATT	AGT	AAT	GGT	AAAT	TGC	AAAT	TGC	TAT	TGC
TTC	CTA	GCT	GCT	ACT	TCT	TCT	TCT	TAT											
1690	1700	1710	1720	1730	1740	1750	1760	1770	1780	1790	1800	1810	1820	1830	1840	1850	1860	1870	1880

AATCATGTTTACAATGGTTACTATCTGCTCATAGACAAACCCATTCTGATTGGATT  
TTAGTACAAAGAATGTTACCAAAATGATAAGCAGGATATCTGTGGTAACTAAA  
1750

TCTTAAATCTGAACCTCATGGCTTACTTCTGCTCATAGACAAACCCATTCTGATTGGATT  
AGAATTAGACTTGAAGTAGCTTGGTAACTAAAATGATAAGCAGGATATCTGTGGTAACTAAA  
1760

1770

TCTTAAATCTGAACCTCATGGCTTACTTCTGCTCATAGACAAACCCATTCTGATTGGATT  
AGAATTAGACTTGAAGTAGCTTGGTAACTAAAATGATAAGCAGGATATCTGTGGTAACTAAA  
1780

1790

TCTTAAATCTGAACCTCATGGCTTACTTCTGCTCATAGACAAACCCATTCTGATTGGATT  
AGAATTAGACTTGAAGTAGCTTGGTAACTAAAATGATAAGCAGGATATCTGTGGTAACTAAA  
1800

1810

1820

1830

1840

1850

1860

1870

1880

NUCLEOTIDE SEQUENCE OF THE RSV F GENE. THE CDNA SEQUENCE IS SHOWN IN THE PLUS (mRNA) STRAND SENSE IN THE 5' TO 3' DIRECTION. THE SIGNAL PEPTIDE (SP) AND THE TRANSMEMBRANE (TM) ANCHOR DOMAIN ARE UNDERLINED. THE PREDICTED F2-F1 CLEAVAGE SITE IS INDICATED BY THE ARROW (↓). AMINO ACIDS DIFFERING FROM THE PUBLISHED PRIMARY SEQUENCE OF THE PROTEIN ENCODED BY THE RSV F GENE ARE BOXED.

18/39

## RESTRICTION MAP OF THE RSV F GENE

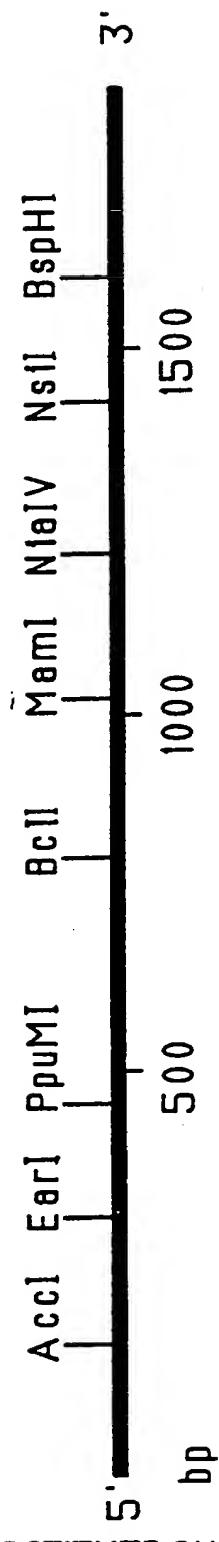


FIG. 6.

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19/39

## FIG.7A. NUCLEOTIDE SEQUENCE OF THE RSV G GENE

MET SER LYS ASN LYS ASP GLN ARG  
 TG CAA AAC ATG TCC AAA A CAAG G ACC AA CG  
 ACG TTT GTAC AGGT TTG TTG CCT GGT TG C  
 10 20 30

THR ALA LYS THR LEU GLU [LYS] THR TRP ASP  
 CAC CG CTAA AG AC ACT AG AAA AG AC CT GG GA  
 GT GGC GATT CT GT GAT CT T T CT GG AC CC CT  
 40 50 60

THR LEU ASN HIS LEU LEU PHE ILE SER SER  
 CACT CT CAAT CATT ATT ATT CAT AT CAT C  
 GT GAG AGT TAG TAA ATA A TAAG TATA G TAG  
 70 80 90 ←

[GLY] LEU TYR LYS LEU ASN LEU LYS SER VAL  
 GGG CTT ATATA AGTT AAAT CTT AAAT CT GT  
 CCC GAAT AT ATT CAATT TAGA ATT TAGACA  
 100 110 120

---

TM —

ALA GLN ILE THR LEU SER ILE LEU ALA MET  
 AGC ACA AAAT CAC ATT CCA TT CATT CT GG CAAT  
 TCG TGT TT AGT GT AA TAG GT AAG AC CC GT TA  
 130 140 150

---

ILE ILE SER THR SER LEU ILE ILE [THR] ALA  
 GATA AT CT CA ACT TC ACT TATA ATT AC AG C  
 CT ATT AG AGT GT GA AG TTA ATT TA AT GT CG  
 160 170 180 →

---

ILE ILE PHE ILE ALA SER ALA ASN HIS LYS  
 CAT CA TATT CA TAG GC CT CG GG CA AA AC CA AA  
 GT AG TATA AGT AT CG GG AG CC GT TT GG TG TT  
 190 200 210

VAL THR [LEU] THR THR ALA ILE ILE GLN ASP  
 AGT CAC ACT A CA ACT G CA AT CA TA CA AG A  
 TC AGT GT GA TG TT GAC GT TAG TAT GT CT  
 2 230 240

---

ALA THR SER GLN ILE LYS ASN THR THR PRO  
 TG CAA ACA AG CAG AT CA AG AA CA CA AC CC  
 AC GT T GT T C ST CT AG TT CT T GT GT TT GG  
 260 270

---

THR TYR LEI THR GLN [ASP] PRO GLN LEU GLY  
 AAC AT AC CT CACT CAG G AT CC CT CAG CT TG  
 TT GT AT GG AG TGA GT CC CT AG GG AG TCG A AC  
 280 290 300

20/39

## FIG.7B.

ILE SER **PHE** SER ASN **LEU** SER GLU ILE THR  
 AATCAGCTTCTCCAATCTGTC TGAAATTAC  
 TTAGTCGAAGAGGTTAGACAGACTTTAATG  
 310 320 330

SER GLN **THR** THR THR ILE LEU ALA SER THR  
 ATCACAAACCAACCACCATACTAGCTTCAAAC  
 TAGTGTGGTGGTGGTATGATCGAAGTTG  
 340 350 360

THR PRO GLY VAL LYS SER **ASN** LEU GLN **PRO**  
 AACACCCAGGAGTCAAGTCAAACCTGCAACCC  
 TTGTTGGTCCTCAGTTCAAGTTGGACGTTGG  
 370 380 390

THR THR VAL LYS THR LYS ASN THR THR THR  
 CACAACAGTCAAGACTAAAAAACACAACAAAC  
 GTGTTGTCAGTTCTGATTTTGTGTTGTTG  
 400 410 420

THR GLN THR GLN PRO SER LYS PRO THR THR  
 AACCCAAAACACAACCCAGCAAGGCCCACTAC  
 TTGGGTTTGTGTTGGTCTGTTGGGTGATG  
 430 440 450

LYS GLN ARG GLN ASN LYS PRO PRO **ASN** LYS  
 AAAAACAAACGCCAAAAACAAACCCACCAACAA  
 TTTTGTGCGGGTTTGTGTTGGTGGTTGTT  
 460 470 480

PRO ASN ASN ASP PHE HIS PHE GLU VAL PHE  
 ACCCAATAATGATTTCACCTCGAAAGTGT  
 TGGTTATTACTAAAAAGTGAAGCTTCACAA  
 490 500 510

ASN PHE VAL PRO CYS SER ILE CYS SER ASN  
 TAACCTTGTAACCCCTGCAAGCATATGCAGCAA  
 ATTGAAACATGGGACGTCGTATACGTCGTT  
 520 530 540

ASN PRO THR CYS TRP ALA ILE CYS LYS ARG  
 CAATCCAAACCTGCTGGGGCTATCTGCACAAAG  
 GTTAGGTTGGACGACCCGATAGACGTTTC  
 550 560 570

ILE PRO ASN LYS LYS PRO GLY LYS LYS THR  
 AATACCAAAACAAAAACCCAGGAAAGAAAC  
 TTATGGTTGTGTTTGTGCTCTTCTTT  
 580 590 600

21/39

## FIG.7C.

THR THR LYS PRO THR LYS LYS PRO THR PHE  
 C A C C A C C A A G C C T A C A A A A A A A C C A A C C T T  
 G T G G T G G T T C G G A T G T T T T T G G T T G G A A  
 610 620 630

LYS THR THR LYS LYS ASP **LEU** LYS PRO GLN  
 C A A G A C A A C C A A A A A A G A T C T C A A A C C T C A  
 G T T C T G T T G G T T T T T C T A G A G T T T G G A G T  
 640 650 660

THR THR LYS **PRO** LYS GLU VAL PRO THR THR  
 A A C C A C T A A A C C A A A G G A A G T A C C C A C C A C  
 T T G G T G A T T T G G T T C C T C A T G G G T G G T G  
 670 680 690

LYS PRO THR GLU GLU PRO THR ILE ASN THR  
 C A A G C C C A C A G A A G G A G C C A A C C A T C A A C A C  
 G T T C G G G T G T C T T C T C G G T T G G T A G T T G T G  
 700 710 720

THR LYS THR ASN ILE **THR** THR THR LEU LEU  
 C A C C A A A A C A A A C A T C A C A A C T A C A C T G C T  
 G T G G T T T G T T G T A G T G T T G A T G T G A C G A  
 730 740 750

THR **ASN** ASN THR THR GLY ASN PRO **LYS** LEU  
 C A C C A A C A A C A C C A C A G G A A A T C C A A A A C T  
 G T G G T T G T G G T G T C C T T T A G G T T T T G A  
 760 770 780

THR SER GLN MET GLU THR PHE HIS SER THR  
 C A C A A G T C A A A T G G A A A C C T T C C A C T C A A C  
 G T G T T C A G T T A C C T T G G A A G G T G A G T T G  
 790 800 810

SER SER GLU GLY ASN **LEU** SER PRO SER GLN  
 C T C C T C C G A A G G C A A T C T A A G G C C C T T C T C A  
 G A G G A G G C T T C C G T T A G A T T C G G G A A G A G T  
 820 830 840

VAL SER THR THR SER GLU **HIS** PRO SER GLN  
 A G T C T C C A C A A C A T C C G A G G C A C C C A T C A C A  
 T C A G A G G T G T T G T A G G C T C G T G G G T A G T G T  
 850 860 870

PRO SER SER PRO PRO ASN THR **THR** ARG GLN  
 A C C C T C A T C T C C A C C C A A C A C A A C A C G C C A  
 T G G G A G T A G A G G T G G G T T G T G T T G T G C G G G T  
 880 890 900

22/39

\*\*\*

G T A G T T A T T A A A A A A A A A A A  
C A T C A A T A A T T T T T T T T T T  
910 920

NUCLEOTIDE SEQUENCE OF THE RSV G GENE. THE cDNA  
SEQUENCE IS SHOWN IN THE PLUS (mRNA) STRAND  
SENSE IN THE 5' TO 3' DIRECTION. THE TRANSMEMBRANE  
(TM) ANCHOR DOMAIN IS UNDERLINED. AMINO ACIDS  
DIFFERING FROM THE PUBLISHED PRIMARY SEQUENCE OF  
THE PROTEIN ENCODED BY THE RSV G GENE ARE BOXED.

FIG.7D.

23/39

## RESTRICTION MAP OF RSV G GENE

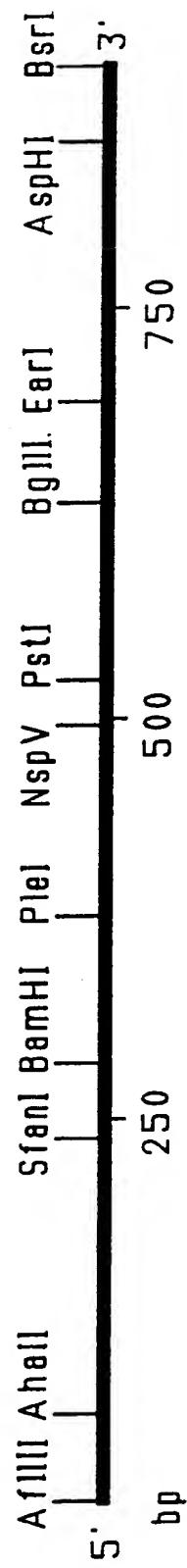


FIG. 8.

24/39

Construction of a Bluescript-based expression vector containing the chimeric F<sub>PIV-3</sub>-F RSV gene with the 5' untranslated region of the PIV-3 F gene intact but lacking the nucleotide sequences coding for the hydrophobic anchor domains and cytoplasmic tails of both the PIV-3 and RSV F genes.

Step 1: Preparation of the plasmid containing the modified PIV-3 F gene  
T7 promoter

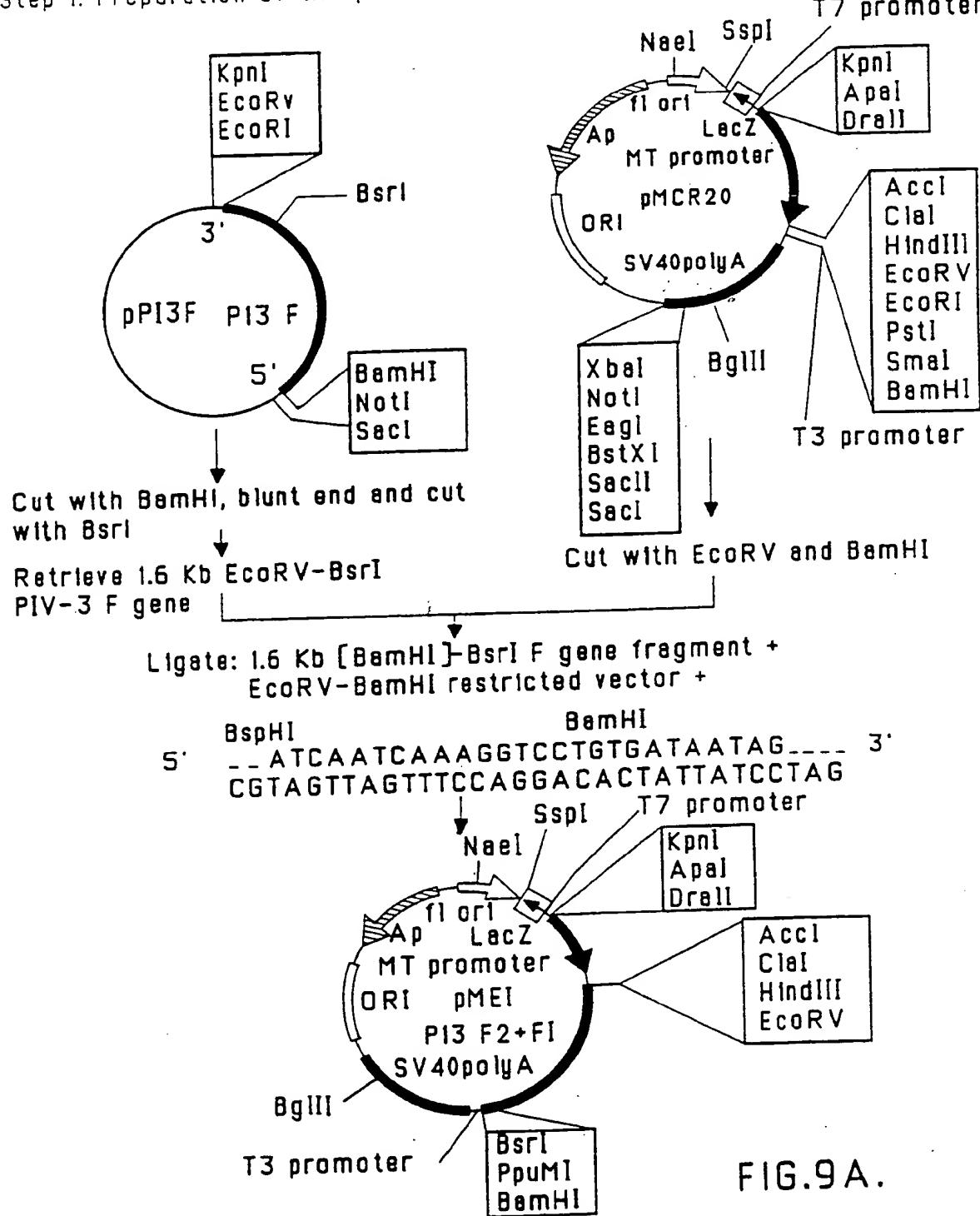
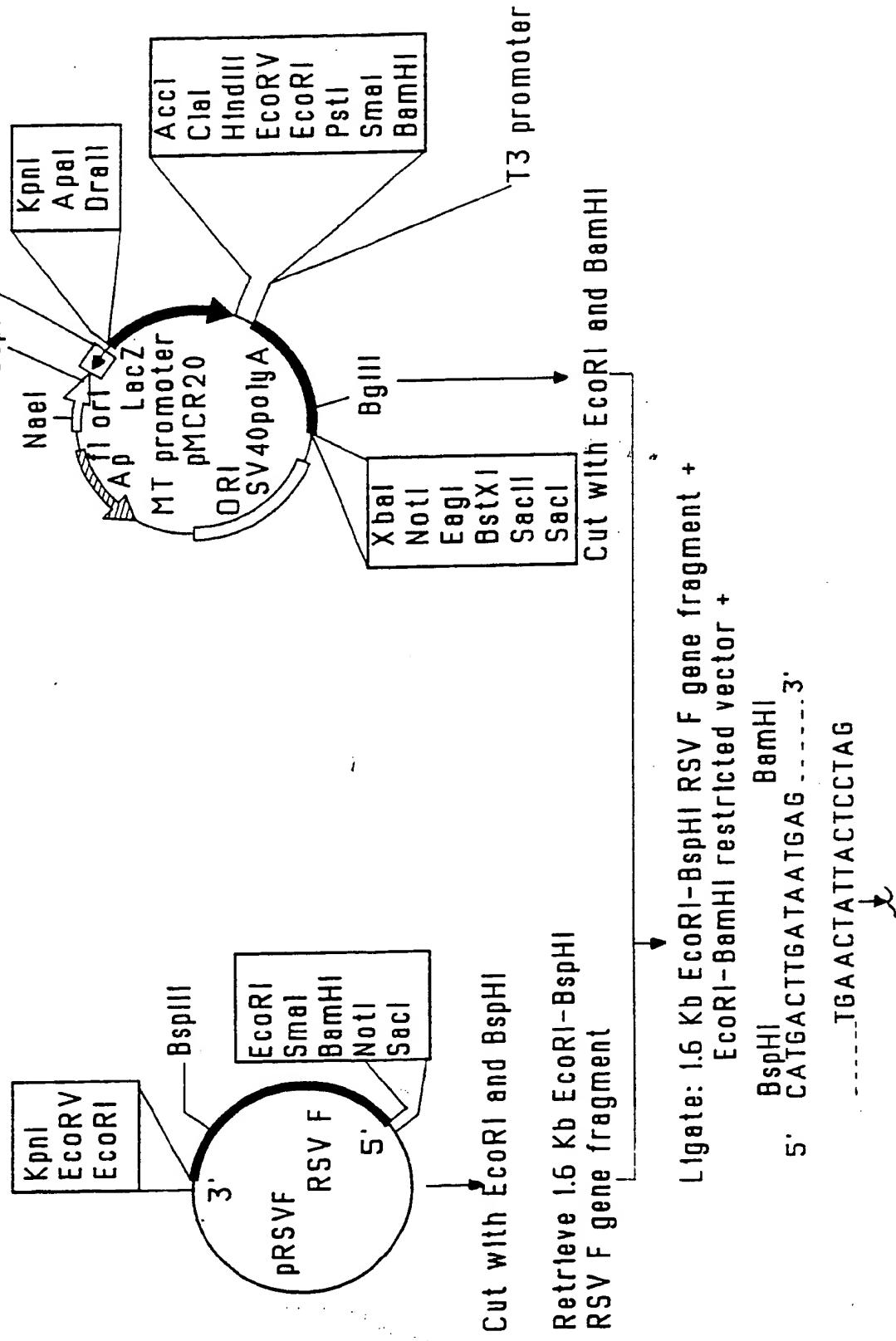


FIG.9A.

25/39

**FIG. 9B.** Step 2: Preparation of the plasmid containing the modified RSV F gene promoter

26/39

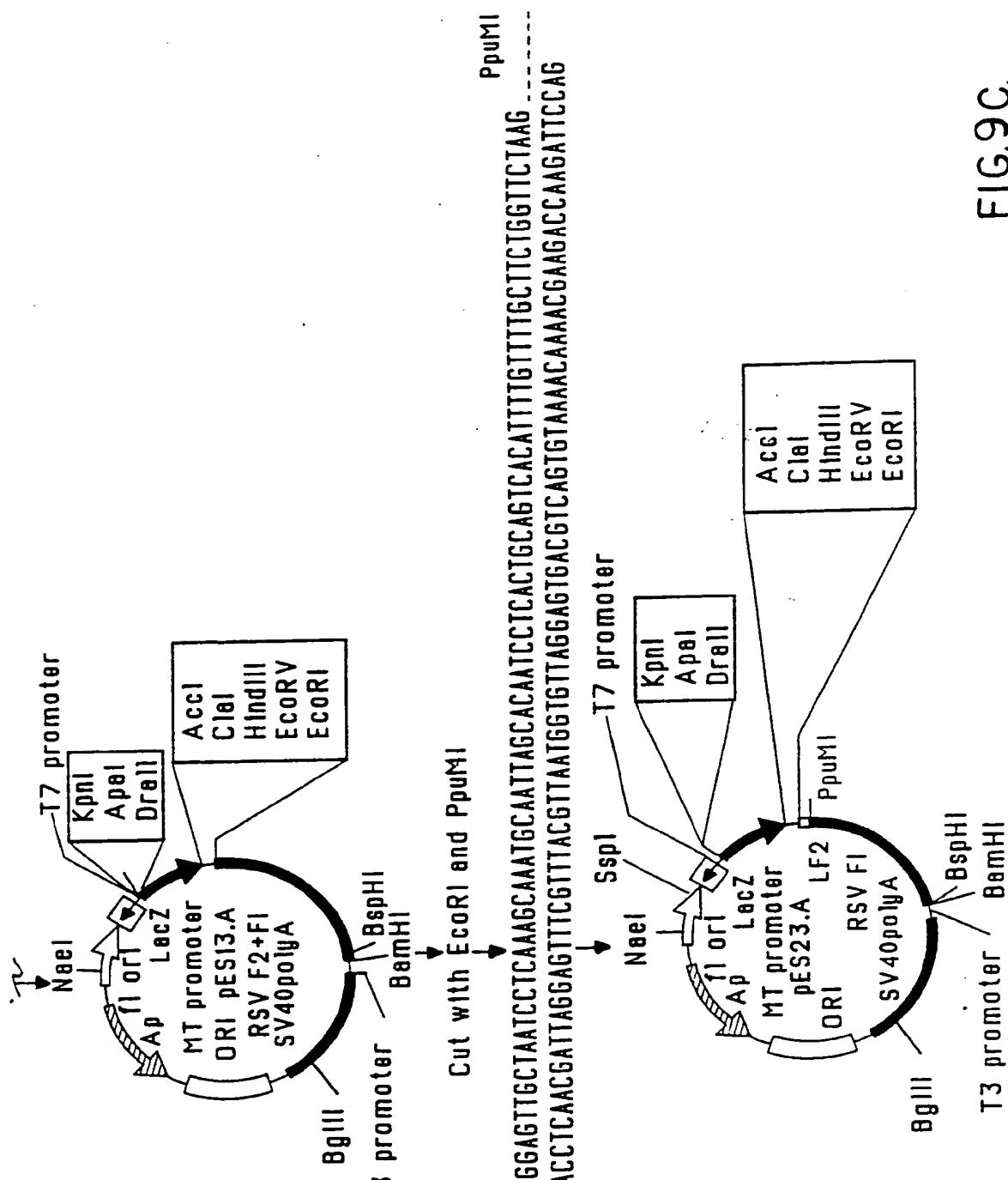
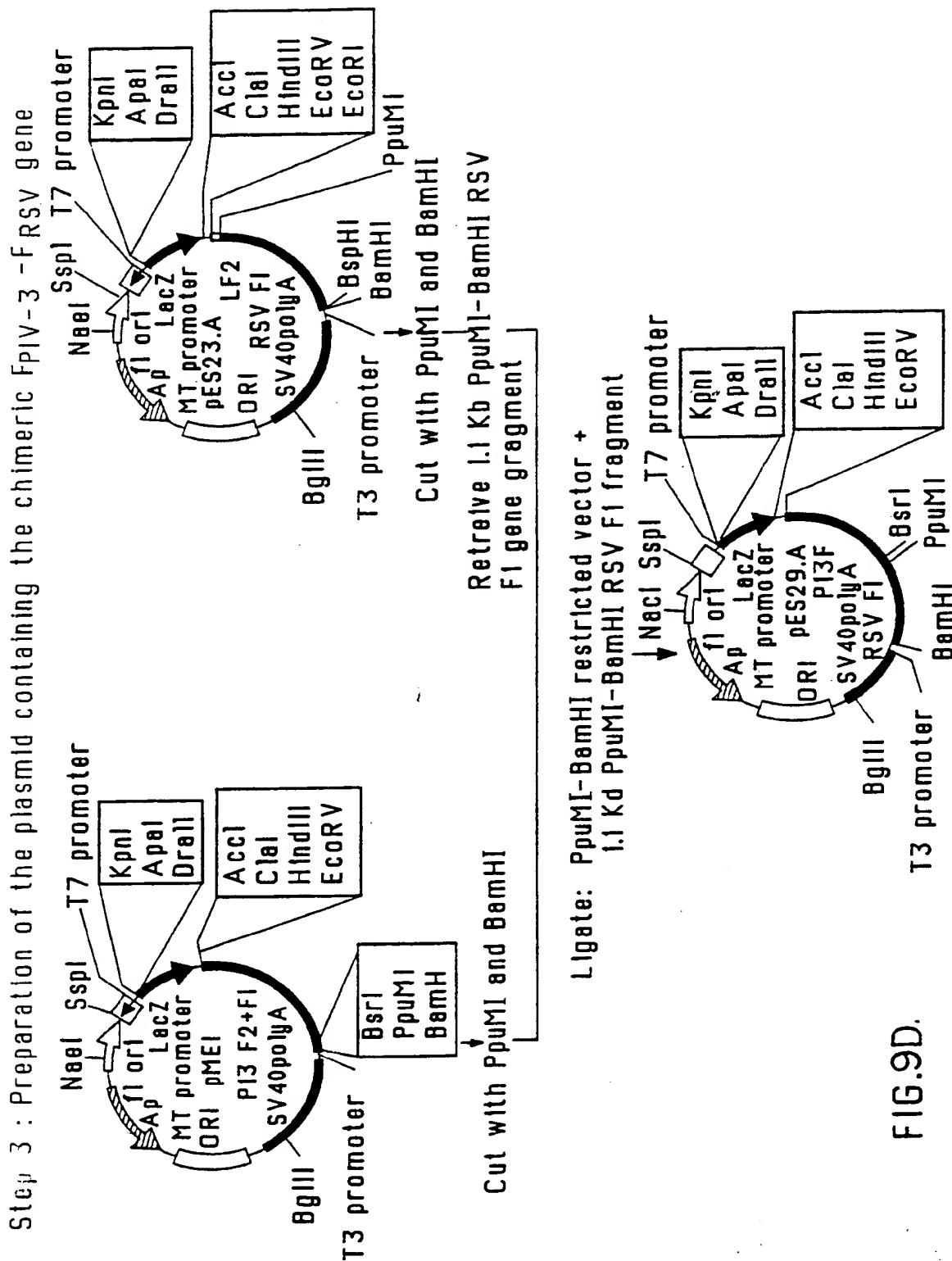


FIG. 9C.

27/39



E. G. D.

28/39

Construction of a Bluescript-based expression vector containing the PIV-3 F gene lacking the 5' untranslated sequence and transmembrane anchor and cytoplasmic tail coding regions.

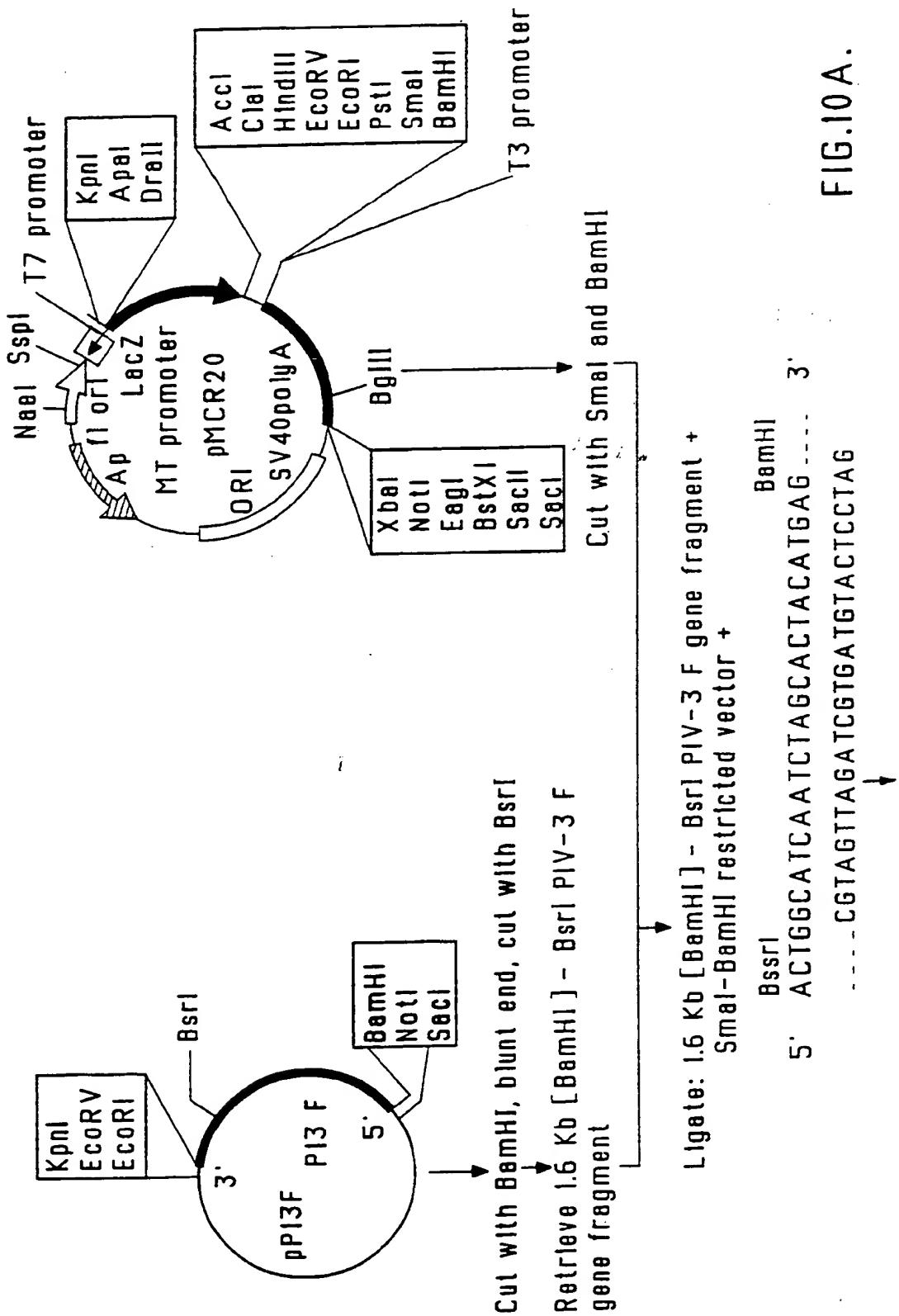
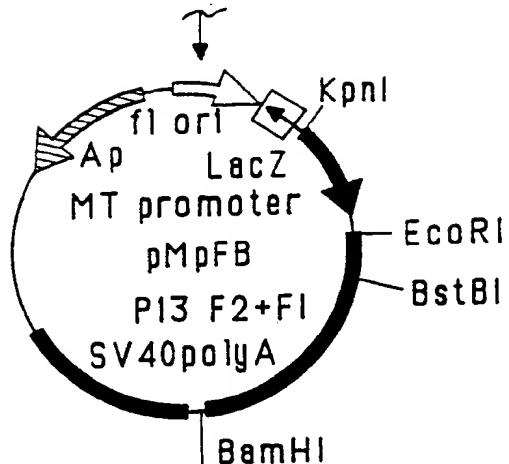


FIG.10 A.

29/39

FIG.10B.



Cut with EcoRI and BstBI

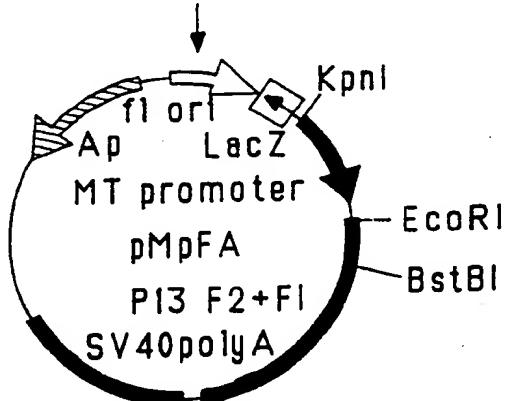
Retrieve: EcoRI-BstBI restricted vector

Ligate: EcoRI-BstBI restricted vector +

EcoRI

AATT CATGCCAACTTAATAC TGCTAATTATTACAACAATGATTATGG  
 CATCTT CCTGCCAAATAGATATCACAAA ACTACAGCAATGTAGGGTGA  
 TTGGTCAACAGTCCCAAAGGGATGAAGATATCACAAA ACTT 3'  
 --- GTACGGTTGAAATTATGACGATTAATAATGTTGTTACTAATACC  
 GTAGAAGGACGGTTATCTATAGTGTGTTGATGTCGTACATCCACATA  
 ACCAGTTGTCAGGGTTCCCTACTTCTATAGTGTGTTGAAGCTT

PpuMI



30/39

Construction of the chimeric F PIV-3-F RSV gene consisting of the truncated PIV-3 F gene devoid of the 5' untranslated region linked to the truncated RSV F1 gene.

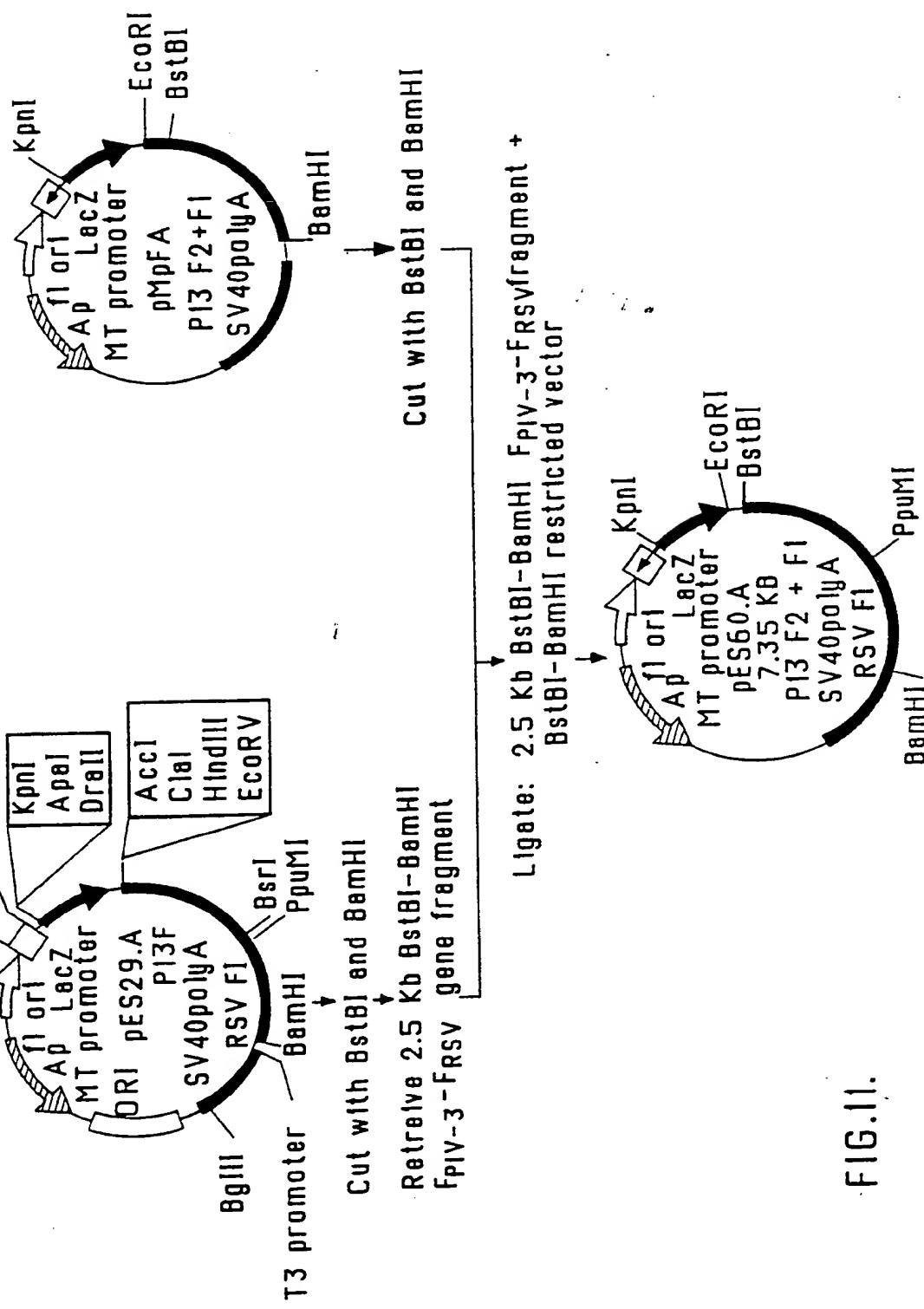
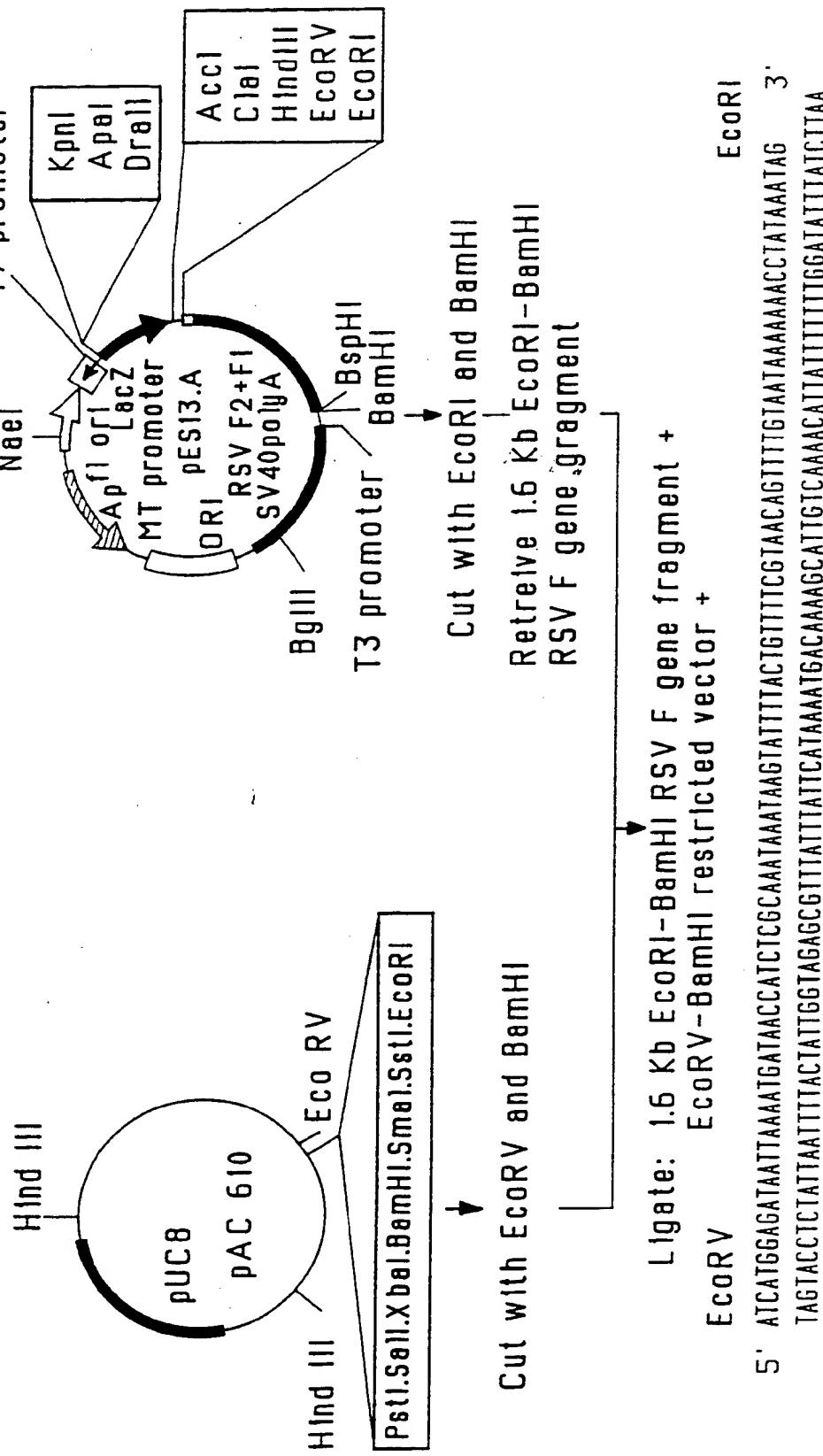


FIG. II.

31/39

**FIG.12A.** Construction of the modified pAC 610 baculovirus expression vector containing the chimeric PIV-3'-RSV gene consisting of the PIV-3' F gene lacking both the 5' untranslated sequence as well as the transmembrane and cytoplasmic tail coding regions linked to the truncated RSV F1 gene T7 promoter



32/39

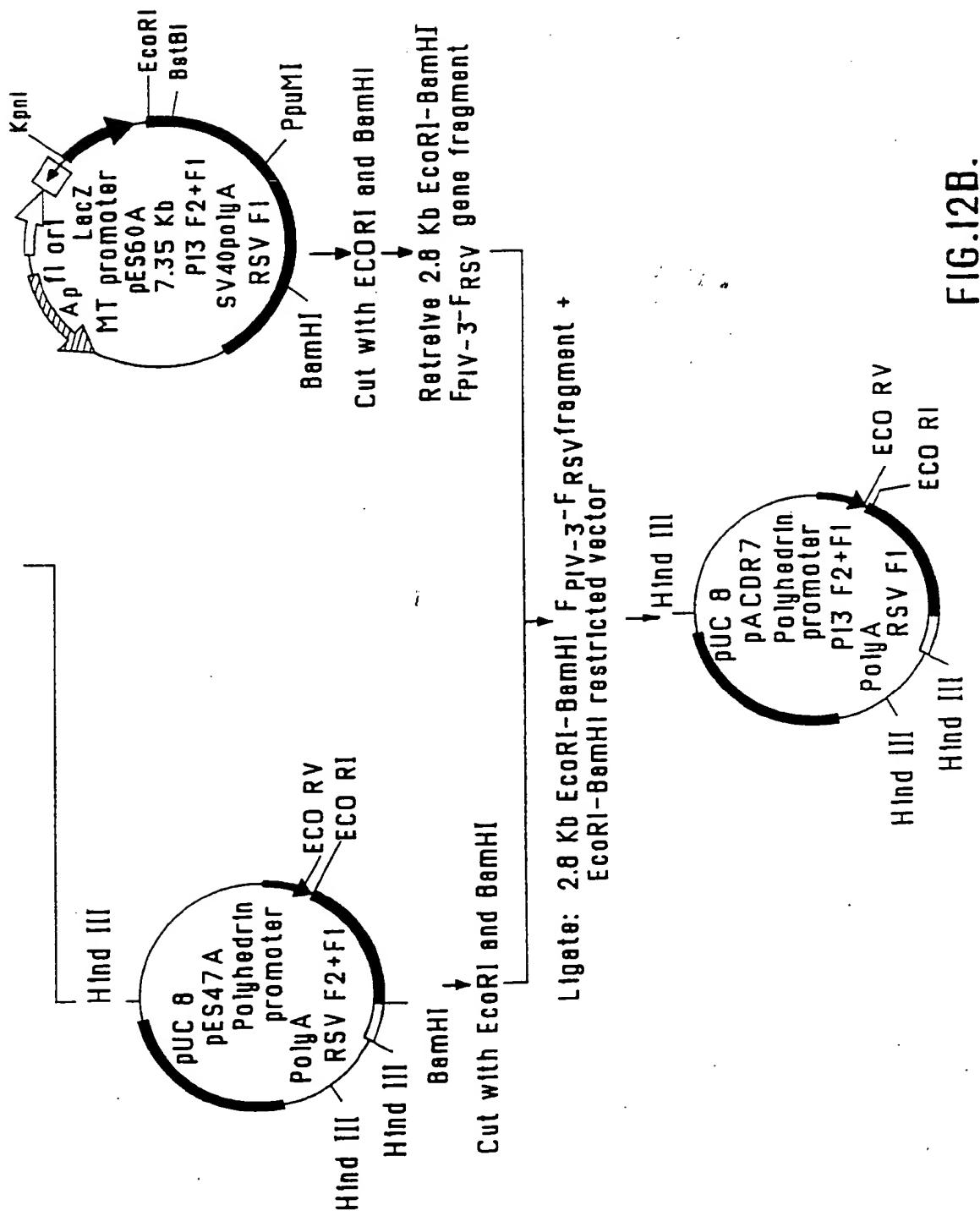


FIG.12B.

33/39

FIG.13

IMMUNOBLOTS OF CELL LYSATES FROM SF9 CELLS  
INFECTED WITH RECOMBINANT BACULOVIRUSES

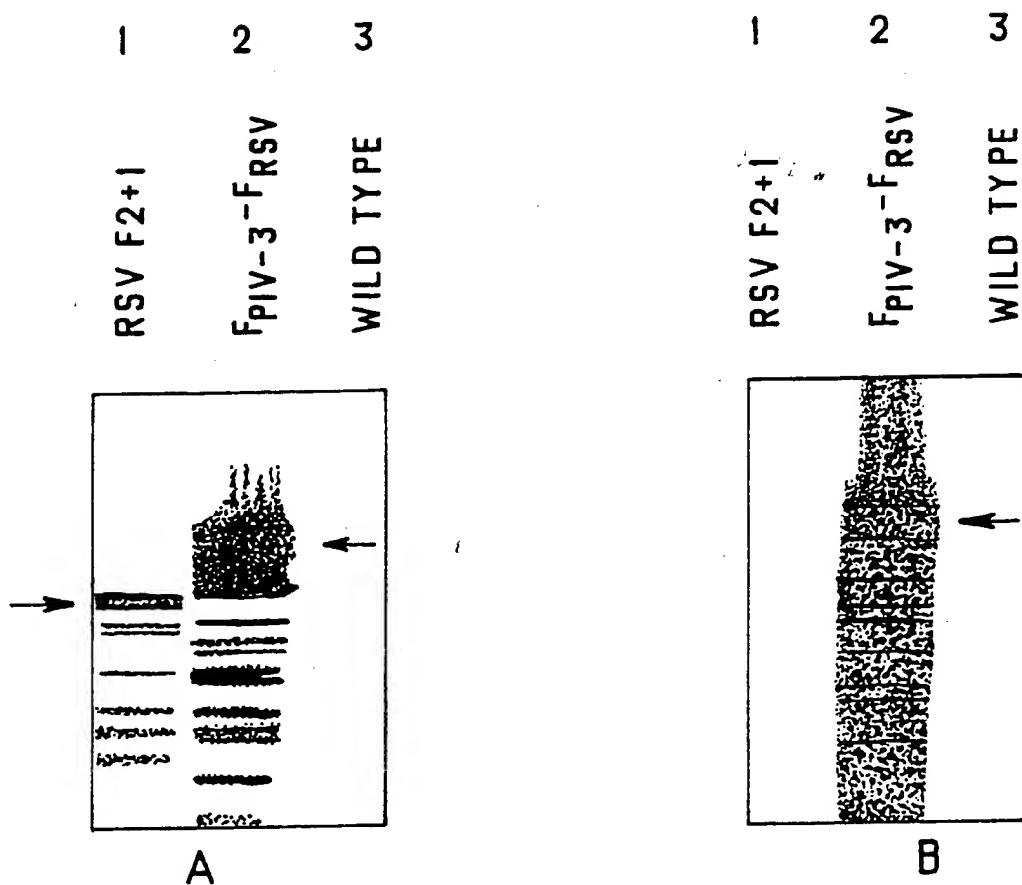
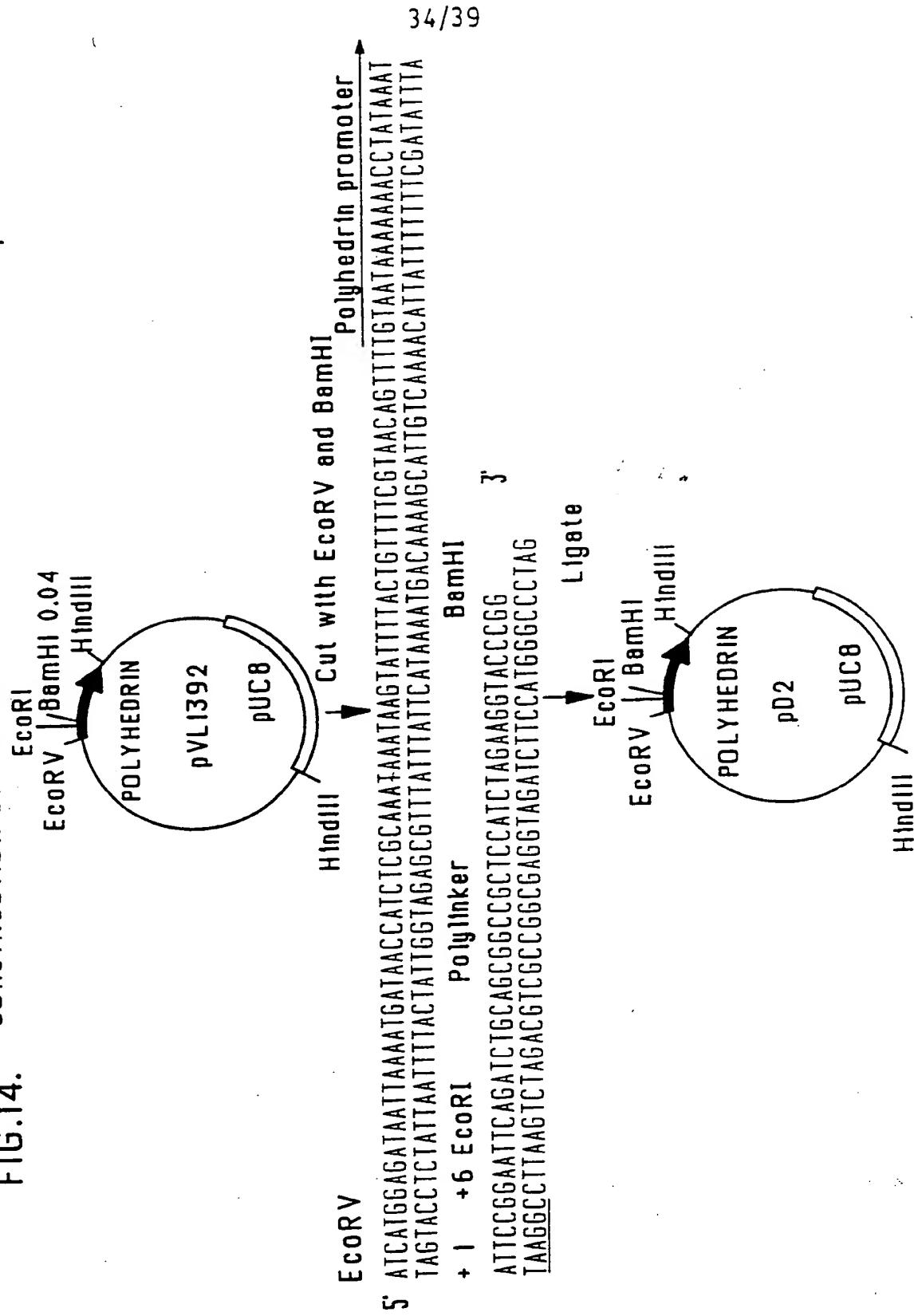


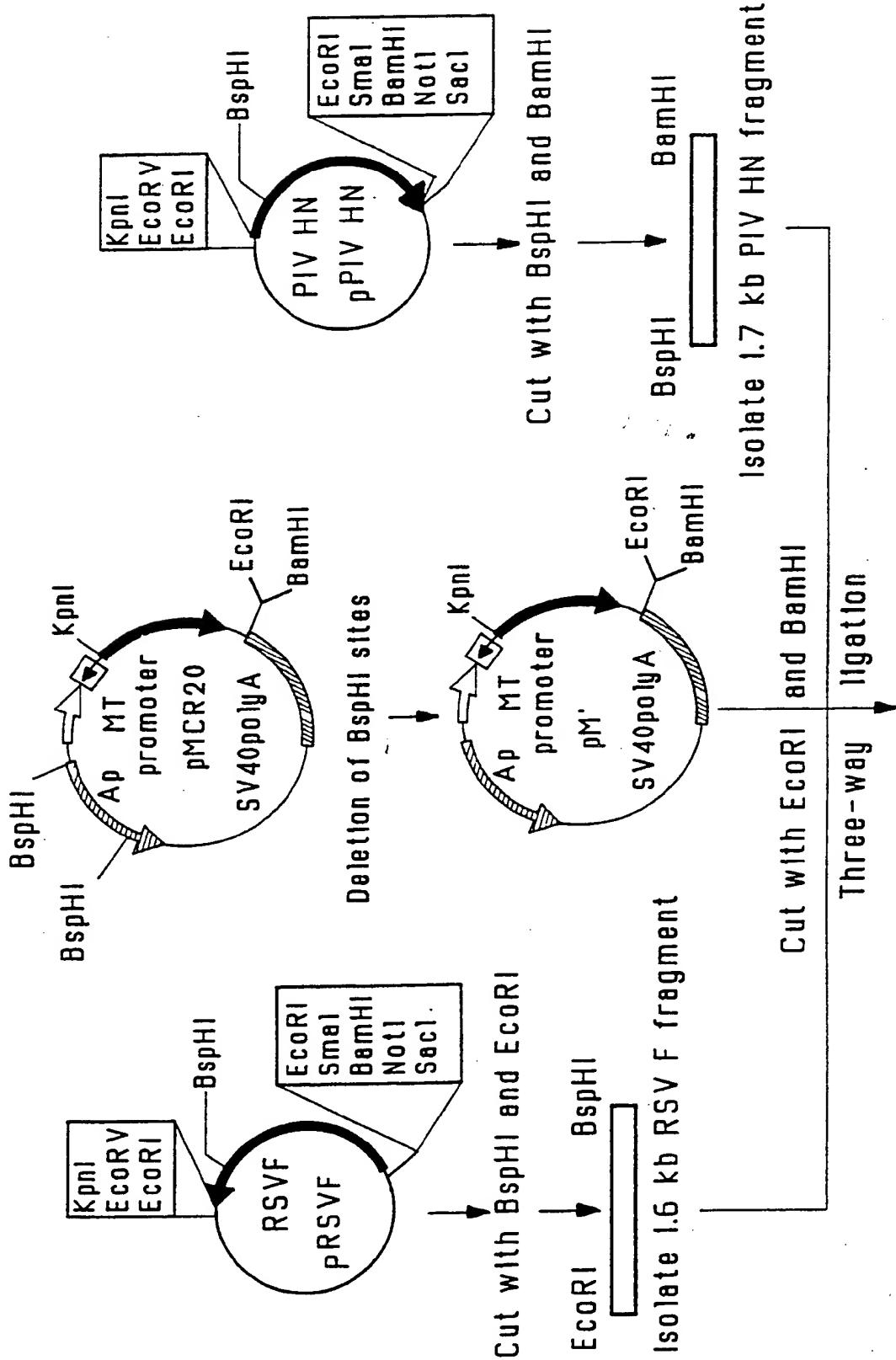
FIG 13 : Immunoblots of cell lysates from SF9 cells infected with recombinant baculoviruses containing the truncated RSV F gene (Lane 1), the chimeric FPIV-3-F RSV gene (Lane 2) or infected with wild type virus (Lane 3) reacted with anti-F RSV Mab (panel A) and anti-F1 PIV-3 antiserum (panel B)

**FIG. 14.** CONSTRUCTION OF THE BACULOVIRUS TRANSFER VECTOR pD2



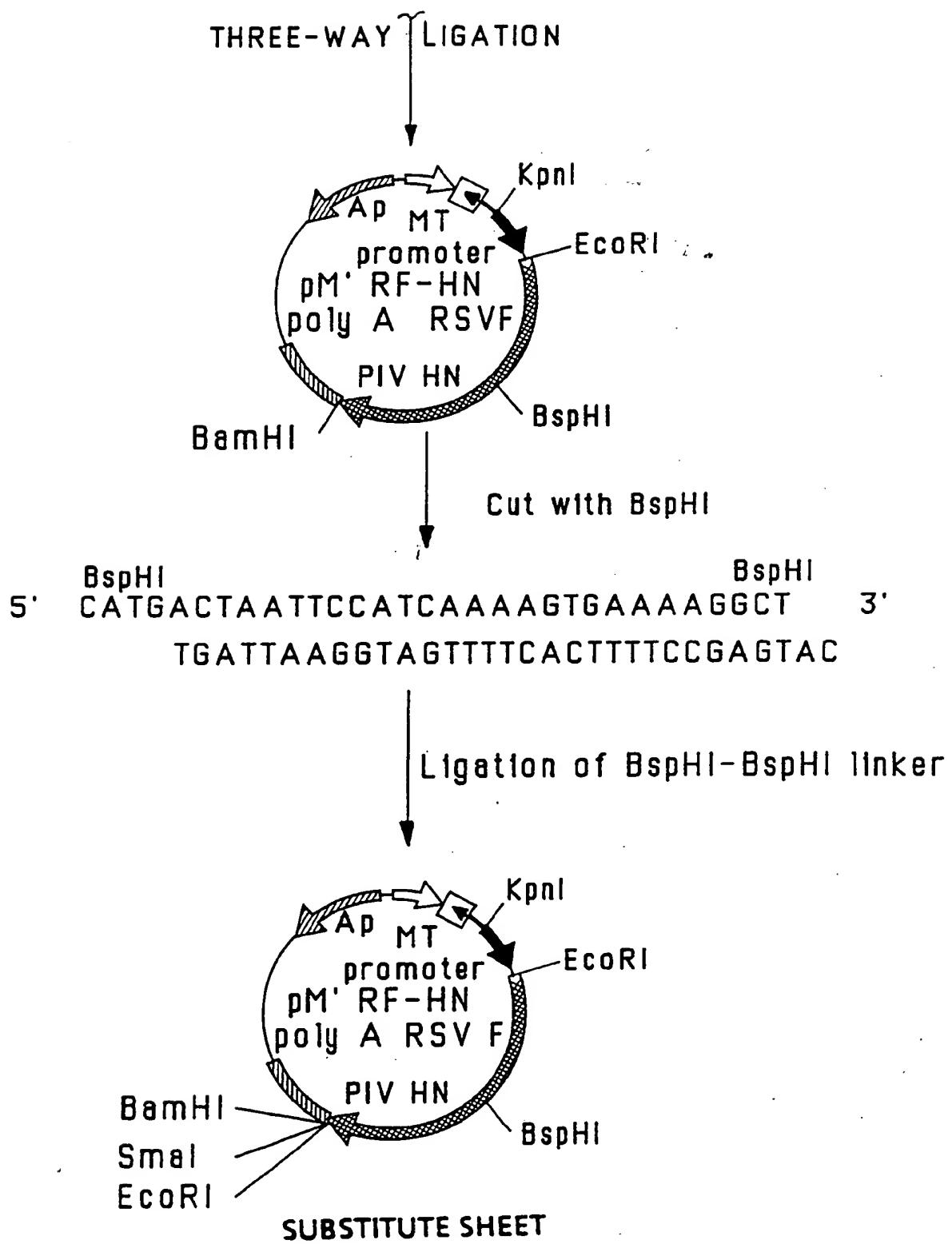
35/39

FIG.15 A. CONSTRUCTION OF THE RSV-HN PIV3 CHIMERIC GENE



36/39

FIG. 15B.



37/39

**FIG.16**

SDS POLY ACRYLAMIDE GEL AND IMMUNOBLOTS OF PURIFIED FRSV-HNPIV-3 CHIMERIC PROTEIN

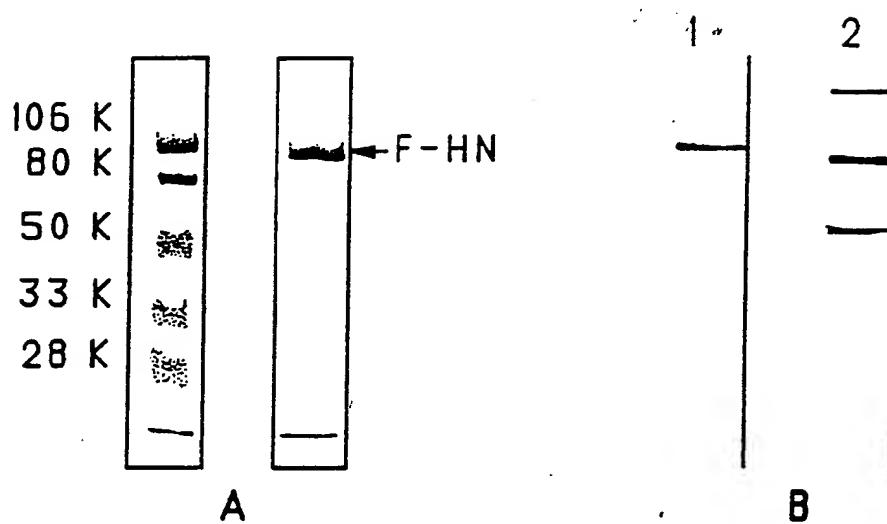
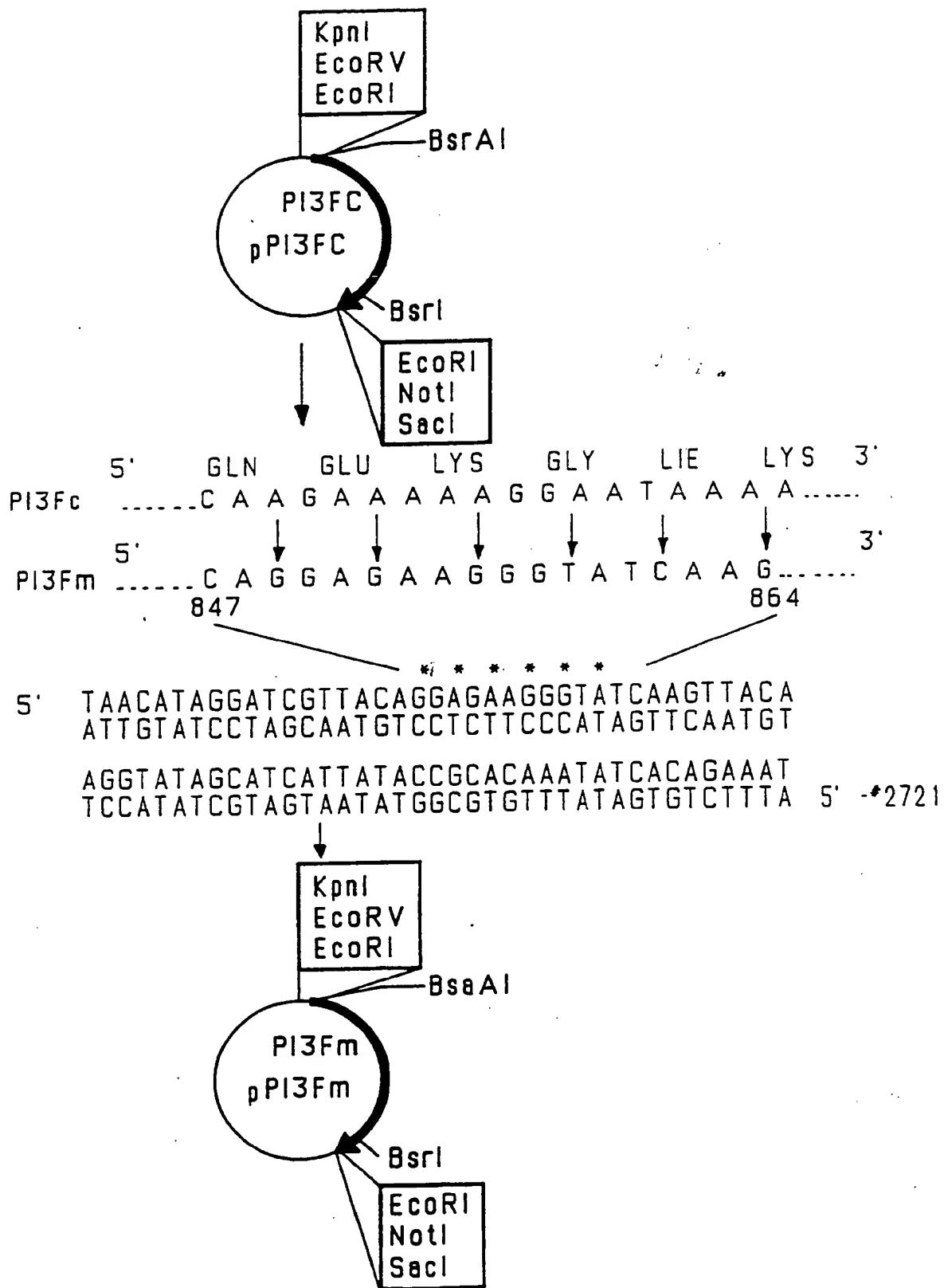


FIG.16 : A) Coomassie-stained SDS polyacrylamide gel of immunoaffinity-purified FRSV-HNPIV-3 protein.

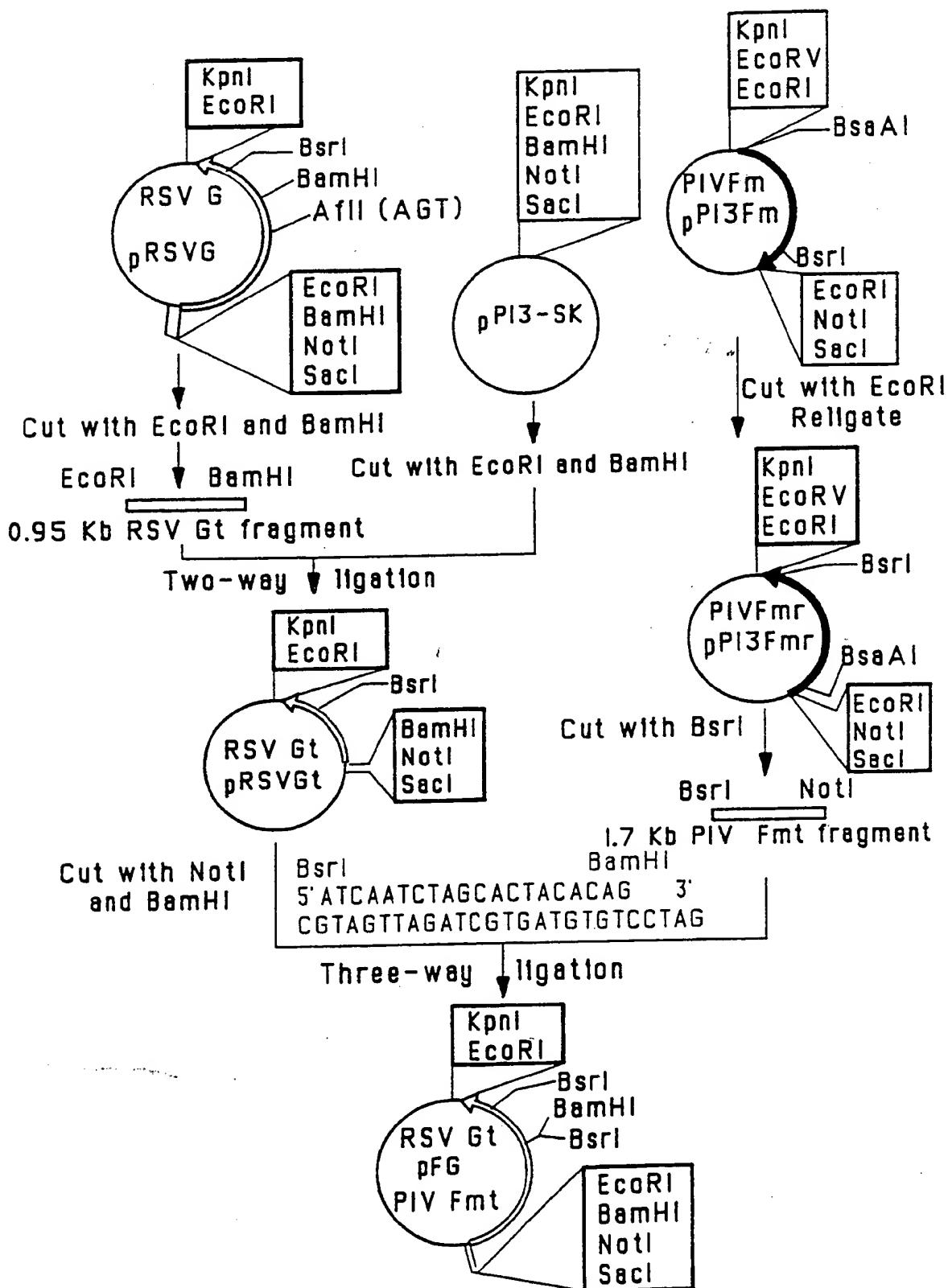
B) Immunoblots of FRSV-HNPIV-3 protein reacted with an anti-F RSV Mab (lane 1) and anti-HN PIV-3 antiserum (lane 2)

38/39

## FIG.17. MUTAGENESIS OF THE PIV-3 F GENE



39/39

FIG.18. CONSTRUCTION OF THE F<sub>PIV3</sub>-G<sub>RSV</sub> CHIMERIC GENE

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 93/00001

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)<sup>6</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC  
 Int.Cl. 5 C12N15/45; A61K39/155; G01N33/569

## II. FIELDS SEARCHED

Minimum Documentation Searched<sup>7</sup>

Classification System	Classification Symbols		
Int.Cl. 5	C12N ;	A61K ;	G01N

Documentation Searched other than Minimum Documentation  
 to the Extent that such Documents are Included in the Fields Searched<sup>8</sup>

III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup>

Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	J. VIROL. vol. 64, no. 8, 1990, pages 4007 - 4012 P. COLLINS 'O glycosylation of glycoprotein g of human respiratory syncytial virus is specified within the divergent ectodomain' see the whole document ---	1-11, 13, 16-26, 28, 29, 32-35, 39-47, 53
X	MOL. CELL. BIOL. vol. 8, no. 4, 1988, pages 1709 - 1714 S. VIJAYA ET AL. 'Transport to the cell surface of a peptide sequence attached to the truncated C terminus of an n-terminally anchored integral membrane protein' see page 1713 ---	1-4, 6, 16-21, 28, 32-36, 39-43, 48-54, 56-58 -/-

<sup>10</sup> Special categories of cited documents :<sup>10</sup>

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

## IV. CERTIFICATION

Date of the Actual Completion of the International Search

13 MAY 1993

Date of Mailing of this International Search Report

03.06.93

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

SKELLY J.M.

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	WO,A,8 910 405 (THE UPJOHN COMPANY) 2 November 1989 cited in the application see the whole document	1-58
Y	WO,A,8 905 823 (THE UPJOHN COMPANY) 29 June 1989 cited in the application see the whole document	1-58
A	J. GEN. VIROL. vol. 70, 1989, pages 2637 - 2644 R. BRIDEAU ET AL. 'Protection of cotton rats against human respiratory syncytial virus' cited in the application	---
A	J. GEN. VIROL. vol. 70, 1989, M. WATHEN ET AL. 'Characterisation of a novel human respiratory syncytial virus chimeric FG glycoprotein' cited in the application	-----

**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/CA93/00001

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 54-56 are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2.  Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1.  As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3.  As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4.  No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

- The additional search fees were accompanied by the applicant's protest.  
 No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

CA 9300001  
SA 68995

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the European Patent Office EDP file on  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 13/05/93

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-8910405	02-11-89	AU-B-	611784	20-06-91
		AU-A-	3197589	24-11-89
		CA-A-	1306709	25-08-92
		EP-A-	0413695	27-02-91
		US-A-	5169628	08-12-92
WO-A-8905823	29-06-89	AU-A-	2785089	19-07-89
		DE-A-	3878468	25-03-93
		EP-A, B	0396563	14-11-90
		US-A-	5194595	16-03-93